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# *The Journal of Laboratory and Clinical Medicine*

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## *CLINICAL AND EXPERIMENTAL*

### THE LANGE TEST\*

#### I. THE INFLUENCE OF PARTICLE SIZE AND HYDROGEN-ION CONCENTRATION OF GOLD SOLS UPON LANGE TEST READINGS ON PARETIC SPINAL FLUIDS

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#### INTRODUCTION

THE Lange test<sup>1</sup> for the clinical diagnosis of certain diseases of the central nervous system, such as paresis, tabes, syphilis, encephalitis, poliomyelitis and meningitis, is not as widely used as its potential possibilities would seem to warrant. This limited use is doubtless due, in part, to the fact that gold sols of the exact quality demanded by the test are extremely difficult to prepare. Furthermore, so little is known definitely about the mechanism of the test and the specific role played by each of the reactants that irregularities that appear when the test is used are not readily interpreted.

The Lange test is used regularly in the Clinical Laboratory at the State of Wisconsin General Hospital with gratifying success. It is regarded as a definite aid in diagnosis and as a valuable means of following the progress of several of the above-mentioned diseases. Its success has been such as to warrant the inception of a program of research, having for its objective a detailed critical study of the test.

The present paper summarizes the results of a series of studies on the influence of particle size and hydrogen-ion concentration of the gold sol on test readings on paretic fluids.

\*From the Department of Chemistry, University of Wisconsin, in collaboration with the Clinical Laboratory of the State of Wisconsin General Hospital, Madison  
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## PREPARATION OF GOLD SOLS

The water used in the preparation of gold sols was purified by distilling it twice in a Pyrex still from a dilute solution of potassium permanganate. Using this water, the following solutions were made up:

- (1) 1 per cent chlorauric acid, by dissolving one 15 grain ampoule of Mallinckrodt's  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  in 100 c.c. of water.
- (2) 2 per cent potassium carbonate, by dissolving 10 gm. of Mallinckrodt's anhydrous potassium carbonate in water to make 500 c.c. of solution.
- (3) 1 per cent formaldehyde, by dissolving 13.5 c.c. of Mallinckrodt's 37 per cent formalin in water to make 500 c.c. of solution.
- (4) 0.01 M resorcinol, by dissolving 1.1 gm. of resorcinol in 1000 c.c. of water.
- (5) 0.2 per cent chlorauric acid, by diluting 20 c.c. of 1 per cent chlorauric acid with 80 c.c. of water.
- (6) 0.4 per cent potassium carbonate, by diluting 20 c.c. of 2 per cent potassium carbonate with 80 c.c. of water.
- (7) 0.2 per cent formaldehyde, by diluting 20 c.c. of 1 per cent formaldehyde with 80 c.c. of water.

A "primary" nuclear sol is prepared as follows:

One hundred cubic centimeters of the cold distilled water are mixed with 5.0 c.c. of 0.4 per cent potassium carbonate and 5.0 c.c. of 0.01 M resorcinol. To this mixture are added 5.0 c.c. of 0.2 per cent chlorauric acid, slowly and with constant stirring. The reduction takes place immediately, an amber-colored sol being formed. When preserved in well-stoppered Pyrex containers, this primary nuclear sol is unchanged over a period of six months.

A "secondary" nuclear sol is prepared as follows:

A mixture of 5 c.c. of 0.4 per cent potassium carbonate and 5 c.c. of 0.2 per cent chlorauric acid is heated just to boiling. One hundred cubic centimeters of cold distilled water are then added. Ten cubic centimeters of the primary nuclear sol are added to this solution and the mixture stirred. Two cubic centimeters of 0.2 per cent formaldehyde are stirred into this mixture and the solution allowed to stand at room temperature until the sol is formed. When preserved in well-stoppered Pyrex containers, this sol is unchanged over a period of six months.

The final sol for use in the test is prepared as follows:

A mixture of 3.5 c.c. of 2 per cent potassium carbonate and 5 c.c. of 1 per cent chlorauric acid is heated just to boiling. Four hundred and eighty cubic centimeters of cold water are added. Into this mixture are stirred 5 c.c.\* of the secondary nuclear sol, followed by 2.5 c.c. of 1 per cent formaldehyde. On standing at room temperature an orange-red sol forms slowly.

\*The volume of "secondary" nuclear sol may be varied, as noted later in this paper.

## INFLUENCE OF THE AVERAGE PARTICLE SIZE OF GOLD SOL ON TEST READINGS

By varying the amount of "secondary" nuclear sol used in the preparation of the final sol, the average particle size can be varied in the manner represented in Table I. Particle size was determined by direct count with the ultra-microscope.

TABLE I

VARIATION OF PARTICLE SIZE WITH VOLUME OF "SECONDARY" NUCLEAR SOLUTION

C.C. OF "SECONDARY" NUCLEAR SOLUTION	AVERAGE PARTICLE SIZE IN MILLIMICRONS
1.25	43
2.5	41
5.0	36
10.0	30
15.0	23.5

It is to be noted that, as the volume of nuclear solution increases, the average particle size decreases. Sols of large particle size were a murky amber yellow, sols with intermediate particles were a clear orange yellow, and sols with very small particles were a brilliant wine red.

Sols of various average particle sizes and constant pH hydrogen-ion concentration were used in Lange tests with samples of spinal fluid from parietic patients. Four sets of test readings, selected at random from a large number taken, are summarized in Tables II to V. All readings in a given table were obtained with the same sample of spinal fluid.

These results all indicate that, for a given pH, as the average particle size of the gold sol increases, the Lange test shows a greater degree of

TABLE II

PARETIC FLUID "A"

pH of Sols = 8.0

AVERAGE PARTICLE SIZE IN MILLIMICRONS	TEST READING
40	5555544321
38	5555543200
35	5555443100
33	3334442100
30	2233421000
23.5	1122100000
17	1122210000
10	0012310000

TABLE III

PARETIC FLUID "B"

pH of Sols = 7.1

AVERAGE PARTICLE SIZE IN MILLIMICRONS	TEST READING
42	55555553
41	5555554
36	55555
30	5555
23.5	555
17	3
10	

TABLE IV  
PARETIC FLUID "L"  
pH of Sols = 7.8

AVERAGE PARTICLE SIZE IN MILLIMICRONS	TEST READING
44	5555555321
39.5	5555554321
36	5555544211
30	5555543100
23.5	5555321000
17	2334442100
10	1221110000

TABLE V  
PARETIC FLUID "AK"  
pH of Sols = 6.3

AVERAGE PARTICLE SIZE IN MILLIMICRONS	TEST READING
40	5555555532
38	5555555422
37	5555555311
30	5555554220
23.5	5555543200
17	5555322100
10	4554221000

precipitation; as the average particle size decreases, the degree of precipitation decreases. In other words, as the average particle size increases, the sensitivity of the sol in the Lange test increases; as the average particle size decreases, the sensitivity decreases. It is thus evident that the gold sol must have the correct average particle size to be satisfactory for use in the Lange

#### THE INFLUENCE OF THE HYDROGEN-ION CONCENTRATION OF GOLD SOL ON TEST READINGS

The pH of the gold sol used in this study can be varied: (1) by varying the amount of potassium carbonate used in its preparation, (2) by varying the amount of formaldehyde used in its preparation, (3) by allowing the sol to stand exposed to the air, (4) by direct addition of dilute hydrochloric acid to the sol, (5) by direct addition of potassium carbonate solution to the sol. Using these five methods, sols of constant particle size and varying pH were made up. These sols were then used in Lange tests with spinal fluid from paretic patients. The results are given in Tables VI to XII.

TABLE VI  
PARETIC FLUID "C"  
Particle Size of Sols = 45 Millimicrons

pH OF SOL	TEST READING
7.57	3555432100
7.27	5555432000
7.01	5555531000
6.83	5555543100
6.63	5555553100
6.49	5555554200

TABLE VII

## PARETIC FLUID "D"

Particle Size of Sols = 42 Millimicrons

pH OF SOL	TEST READING
7.07	3444310000
7.32	5554321000
6.92	5555432100
6.50	5555543210
6.34	5555554300
6.10	5555554310

TABLE VIII

## PARETIC FLUID "E"

Particle Size of Sols = 37.5 Millimicrons

pH OF SOL	TEST READING
7.56	2233321000
7.21	3334321000
6.81	555442100
6.40	5555432100
6.01	5555543210

TABLE IX

## PARETIC FLUID "F"

Particle Size of Sols = 38.5 Millimicrons

pH OF SOL	TEST READING
7.37	1233100000
6.88	3333100000
6.53	5554210000
6.05	5554321000
5.72	555543210
5.54	5554320000
5.10	5555421000
4.96	5555543210
4.38	5555554333

TABLE X

## PARETIC FLUID "G"

Particle Size of Sols = 27 Millimicrons

pH OF SOL	TEST READING
8.21	2223210000
7.15	5333210000
6.57	5554210000
6.12	5554310000
5.71	5554321000
5.64	5555542100
5.36	5555421000
5.10	5554210000
5.06	5554431000
4.56	5555543100

The results show very definitely that, as the pH of the sol decreases, its sensitivity in the Lange test increases. It is thus obvious that the pH of a gold sol must be carefully controlled if it is to be satisfactory for use in the Lange test.

## DISCUSSION

It is to be noted that pH and particle size may exert a compensating effect in producing a gold sol suitable for use in the Lange test. Thus, whereas increase in particle size gives a more sensitive sol, increase in pH gives a more stable sol. There are, theoretically, an unlimited number of pH-particle size combinations, all of which give a sol of the same sensitivity. Practically, however, there is a limit to these combinations because if the particle size goes above 40 millimicrons, the sols are too murky to be suitable; if the pH goes below 5, the readings tend to become erratic (see Tables XI and XII).

TABLE XI  
PARETIC FLUID "H"  
Particle Size of Sols = 24 Millimicrons

pH OF SOL	TEST READING
7.40	3344321000
6.60	5554321000
5.80	5555432100
5.06	5555433100
4.60	5555543200
4.42	5555543343

TABLE XII  
PARETIC FLUID "I"  
Particle Size of Sols = 17.5 Millimicrons

pH OF SOL	TEST READING
7.35	3333100000
6.18	5554210000
5.77	5554210000
5.30	5555421000
4.56	5555541000
4.49	5555432310
4.10	555555533
3.96	555555554

The fact that pH and particle size exert this compensating effect is, perhaps, a part of the explanation for the fact that so many different techniques, as practiced in different laboratories, all yield gold sols suitable for use in the Lange test. Also, the fact that both pH and particle size are so very sensitive to very slight variations in technique and, particularly, to variations in the concentration and purity of reagents used will explain why sols of the exact quality required in the Lange test are so difficult to prepare.

There probably is an optimum combination of pH and particle size which will give an ideal Lange test gold sol. Just what this optimum combination may be is still a question, although the results obtained to date seem to indicate that sols of small particle size, with relatively low pH (about 6) are more satisfactory than sols of larger particles and higher pH.

The explanation of why the sensitivity of a gold sol is dependent upon particle size and pH would appear to be fairly simple. It is a well-known fact in colloid science that, other things being equal, larger particles are less stable than smaller particles. Hence, an increase in particle size should give a more sensitive sol. Gold sols are negatively charged, due, doubtless, to

adsorption of negative ions. Hence, an increase in the concentration of negative hydroxyl ions (increase in pII) should increase the stability of the sol.

Preliminary studies indicate that the Lange test readings in tabes, syphilis, encephalitis, spinal meningitis, tubercular meningitis and poliomyelitis are influenced by the pII and particle size of the gold sol in the same manner as are the readings in paresis. Detailed data on studies with these diseases will be reported in a later paper.

#### SUMMARY

The sensitivity of gold sols in the Lange spinal fluid test for paresis increases with an increase in particle size and decreases with an increase in pH.

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The authors wish to express their indebtedness to Mr. R. E. Dover and Mr. Ralph Walker, of the Neuropathological Laboratories of the Wisconsin General Hospital, for their help, their valuable suggestions, and their helpful criticisms.

## THE EFFECT OF FEVER THERAPY UPON CARBOHYDRATE METABOLISM\*

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IT IS known that infectious fevers cause moderate degrees of hyperglycemia. Simpson,<sup>1</sup> and Hench and Slocumb,<sup>2</sup> among others, have observed the same phenomenon in artificial hyperthermia. According to these observers, the hyperglycemia is due to blood concentration which occurs during fever therapy.

We have also noted hyperglycemia during fever therapy (see Table I), but we differ in the interpretation of the phenomenon and feel that its comprehension is of theoretical as well as of practical significance. We are ready to concur in the statement that loss of fluids during hyperthermia generally causes an increased concentration of blood constituents, such as nonprotein nitrogen (see Table I), urea, etc. However, to attribute the increase in blood sugar to concentration contradicts the physiology of the regulation of the glycemic level.

It is known that when the sugar content of the blood is augmented, e.g., by the intravenous injection of glucose, the resultant hyperglycemia is only of short duration, and the blood sugar soon returns to its original level. This is brought about by a regulatory mechanism in the liver which acts by changing the velocity relationship of a reversible reaction. This reversible enzymatic reaction consists of glycogen storage in one direction and glycogen

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breakdown in the opposite direction. The blood sugar level per se is the primary factor influencing the relationship between the velocities of these two opposite processes. Whenever the concentration of the blood sugar rises above the postabsorptive (fasting) level, sugar is rapidly withdrawn from circulation due to an increase of the reaction velocity in the direction of glycogen storage. When, for example, one injects 1.0 to 1.5 gm. of glucose per kg. body weight into the blood stream of a dog, the ensuing hyperglycemia disappears within thirty minutes.

The regulatory mechanism is exquisitely fine and responsive in its tendency to maintain a constant blood sugar level. This ready adaptability makes it possible for the blood sugar to remain almost immutable in the post-absorptive state. Although glucose is constantly withdrawn from the circulation by the peripheral tissues, the liver replaces it at the same rate that it is withdrawn. The rate of glycogenolysis is so astonishingly adaptable to the changing requirements that even during moderate physical exercise (when glucose is withdrawn from the blood stream at an increased rate) no demonstrable changes in the blood sugar level occur. Therefore, one is compelled to raise the question, why, if this fine regulatory mechanism so efficaciously disposes of hyperglycemias resulting from the injection of glucose, should it fail to take care of the relatively slight and gradual increases in glucose concentrations occurring during artificial fever. Thus all that is known concerning the mechanism which regulates the blood sugar level is in direct conflict with the assumption that hyperglycemia during fever therapy is due to blood concentration.

TABLE I

EFFECT OF ARTIFICIAL FEVER THERAPY UPON BLOOD SUGAR, NONPROTEIN NITROGEN AND PHOSPHORUS

8

Temperature Range—104-106R. Duration of Fever 3-6 Hours.

Apparatus: Kettering Hypertherm

SUGAR		N.P.N.		PHOSPHORUS	
MG. %		MG. %		MG. %	
BEFORE	R	BEFORE	R	BEFORE	R
84	92	27	33	---	---
89	93	19	22	---	---
87	109	--	--	---	---
88	94	26	30	2.9	2.1
84	87	28	38	2.7	1.9
97	98	24	27	2.5	2.2
80	101	16	21	---	---
88	93	--	--	---	---
83	95	15	14	---	---
95	100	20	18	---	---
92	105	20	22	---	---
83	115	--	--	3.4	1.5
86	87	--	--	3.2	1.5
69	85	16	19	2.6	1.3
81	93	20	23	3.6	2.4
86	93	--	--	3.1	2.7
87	89	18	30	4.8	4.8
84	88	20	17	3.5	2.9
82	82	16	16	3.8	3.7
80	85	17	24	4.0	3.8
80	85	15	20	4.1	3.8

Each horizontal line represents one patient.

An additional contradiction to the concentration theory is found in the fact that the inorganic blood phosphates show a substantial drop during hyperthermia. Our results (see Table I) show this fall in phosphorous and correspond to those reported by Bischoff,<sup>3</sup> Daly,<sup>4</sup> and others. The decrease in inorganic phosphates is readily explained by an increased rate of glucose utilization, due to a markedly increased metabolic rate during fever. Inorganic blood phosphates invariably decrease whenever increased utilization of glucose occurs (as after insulin administration, after the ingestion of glucose and during exercise). This adequately accounts for the drop in inorganic blood phosphates during fever therapy.

The increased rate of glucose utilization also explains the hyperglycemia during elevated body temperatures. Loseke and Gunderson<sup>5</sup> have reported marked depletion of liver glycogen in rabbits undergoing artificial fever. Somogyi<sup>6</sup> has emphasized in a recent paper that whenever blood sugar is utilized by body tissues at an increased rate (such as after insulin injection, during exercise and during hyperthermia), the glycogen reserve of the liver becomes depleted. This is due to the fact that the accelerated withdrawal of blood sugar by the peripheral tissues causes an increased rate of glycogen breakdown in the liver, so that the sugar content of the blood may be restored to its normal level. This compensatory process frequently overshoots its aim and hyperglycemia results. Individual differences in this regulatory mechanism explain the variations in the degree of the hyperglycemia encountered. In some instances the hyperglycemia is slight; in others the blood sugar rises as much as 20 to 30 mg. per cent above the fasting level. Therefore, we conclude that blood concentration is not the cause of the hyperglycemia during artificial fever.

This concept is of practical significance in fever therapy. Due to the increased combustion of sugar, fever tends to deplete the glycogen reserve of the body tissues, especially that of the liver. This depletion may become so excessive as to result in ketosis. Such an occurrence has been brought to our attention through a personal communication from Dr. H. W. Kendall, of the Kettering Institute, Dayton, Ohio. The depletion, even if it does not lead to ketosis, is certainly undesirable. Because of this, we feel that it is of considerable value to prepare patients for fever therapy by giving them liberal carbohydrate diets between sessions. In order to avoid a gain in weight to which these patients show a distinct tendency, we suggest a moderation of fats.

The preparation of diabetic persons for fever therapy is especially pertinent. One of the important pathologic factors in diabetes is the tendency toward an increased rate of glycogenolysis in the liver. As Somogyi has recently emphasized, if any factor which stimulates glycogenolysis (such as artificial fever) is superimposed upon this, the result may be excessive hyperglycemia, glycosuria, and frequently ketosis. (This may also explain the response of persons with diabetes to infectious fevers.) The vicious process can be combatted by means of adequate administration of carbohydrates and by inhibiting the rate of glycogenolysis with insulin. We feel that this concept should be applied to diabetic patients, when subjected to artificial fever.

## CONCLUSION

Hyperglycemia during fever therapy is not due to blood concentration, but to disturbances in carbohydrate metabolism. This concept may have an important bearing upon the preparation of patients for fever therapy.

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## IS THERE A PLATELET-REDUCING SUBSTANCE IN THE SPLEEN OF THROMBOCYTOPENIC PURPURA?\*

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TROLAND and Lee<sup>1</sup> in January, 1938, described a platelet-reducing substance in the spleen of patients suffering from thrombocytopenic purpura. Later in July of the same year, they described their findings in detail and reported that extracts from three spleens from patients suffering from thrombocytopenic purpura contained a substance which produced a marked fall in the number of blood platelets in rabbits. They described falls as great as 605,000 per c.mm. to 100,000 per c.mm. in twenty-four hours. Not long after the first report we had the opportunity to study the spleens removed from two patients suffering from thrombocytopenic purpura. We delayed our report somewhat, hoping that additional cases would be obtained. Although we have seen some additional patients during the past year splenectomy was not recommended or carried out in any of them.

Patient M. G., female, aged 19 years, was admitted to the hospital February 1, 1938. She presented the typical history and findings of thrombocytopenic purpura. She had numerous petechiae over the legs and arms, her spleen was enlarged, and her blood platelet count varied from 85,000 to 50,000. The patient was operated upon February 22. The spleen was removed, and was found to measure 12 by 3.6 by 3 cm.; it weighed 170 gm., had a smooth surface, distinct notching, and no adhesions. Following the operation the patient's platelet count rose to 150,000 on February 25, and on March 5 it reached 350,000. The patient has had no further purpuric symptoms.

\*From the Hixon Laboratory of Medical Research, University of Kansas School of Medicine, Kansas City.

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The extract from the spleen was prepared in the manner described by Troland and Lee in their first article. The spleen was taken directly from the operating room and ground in a food chopper. Twice its volume by weight of acetone was added and the mixture allowed to stand in a sealed jar. After seventeen days the supernatant fluid was filtered off and collected, and the acetone evaporated off, leaving a thick brownish material. This material was dissolved in 125 c.c. of normal saline solution. Table I shows the effect of this solution on intravenous injection.

TABLE I

DATE	FIRST COUNT	SECOND COUNT	REMARKS
<i>Rabbit No. 1</i>			
2/27/38	460,000	500,000	
3/ 2/38	470,000	480,000	
3/ 4/38	420,000	440,000	
3/ 9/38	500,000	440,000	10:00 A.M. 10 c.c. spleen extract intravenously
3/10/38	590,000	530,000	10:00 A.M.
	510,000	520,000	5:00 P.M.
3/11/38			8:30 A.M. 20 c.c. spleen extract intravenously
	780,000	750,000	1:30 P.M.
	630,000	580,000	5:30 P.M.
3/12/38	480,000	410,000	8:30 A.M.
<i>Rabbit No. 2</i>			
3/18/38	500,000	480,000	
3/19/38	480,000	480,000	
3/21/38	560,000	510,000	11:15 A.M. 20 c.c. spleen extract intravenously
3/21/38	580,000	620,000	5:20 P.M.
3/22/38	400,000	440,000	5:00 P.M.
3/23/38	410,000	450,000	10:20 A.M.
<i>Rabbit No. 3</i>			
3/18/38	440,000	500,000	
3/19/38	460,000	520,000	
3/21/38	510,000	540,000	11:15 A.M. 10 c.c. spleen extract intravenously
	550,000	550,000	5:20 P.M.
3/22/38	510,000	510,000	3:00 P.M.

The platelet counts were made by the method of Rees-Ecker. The results of these observations are not at all conclusive, the blood showing at times an increase, rather than a decrease, following injection.

The second patient, D. T., a girl 5 years of age, presented the typical history and clinical appearance of purpura hemorrhagica. The patient's platelet counts before operation varied from 80,000 to 90,000. The patient was operated upon May 18, 1938. The spleen weighed 89 gm. and measured 11 by 6.5 by 2 cm. in size. Following the operation the patient's count was 122,000 on May 22, and during the last week in the hospital it remained above 250,000.

The extract from the spleen of the second patient was prepared in a manner similar to that of the first patient. The ground-up spleen was allowed to remain in a sealed jar with the acetone for twenty-one days. As the spleen was smaller the brownish material was taken up in a smaller quantity of fluid, only 60 c.c. of salt solution being employed. The results of these tests are shown in Table II.

TABLE II

DATE	FIRST COUNT	SECOND COUNT	REMARKS
<i>Rabbit No. 2</i>			
6/ 7/38	580,000	540,000	8:45 A.M. 20 c.c. spleen extract intravenously
	500,000	460,000	2:45 P.M.
6/ 8/38	540,000	520,000	3:45 A.M.
	790,000	810,000	10:30 A.M. 20 c.c. spleen extract intravenously
6/22/38	480,000	500,000	6:00 P.M.
	380,000	350,000	10:30 A.M.
6/24/38	700,000	750,000	10:30 A.M.
<i>Rabbit No. 4</i>			
6/22/38	730,000	700,000	10:30 A.M. 20 c.c. spleen extract intravenously
	560,000	520,000	6:00 P.M.
6/23/38	570,000	550,000	10:30 A.M.
6/24/38	600,000	650,000	10:30 A.M.

The splenic extract from this patient apparently produced a definite fall in platelet count. The figures, however, are not nearly so striking as those reported by Troland and Lee. In Table III are the platelet counts obtained in rabbits injected with normal spleen extract, extract from the spleen in Banti's disease, and extract from the spleen in myelogenous leucemia.

TABLE III

DATE	FIRST COUNT	SECOND COUNT	REMARKS
<i>Rabbit No. 1</i>			
4/ 6/38	610,000	600,000	9:30 A.M. 20 c.c. normal spleen extract intravenously
	650,000	620,000	3:30 P.M.
4/ 8/38	570,000	540,000	9:30 A.M. 30 c.c. extract intravenously
	580,000	610,000	3:30 P.M.
<i>Rabbit No. 2. Banti's Disease</i>			
5/ 9/38	610,000	580,000	
5/10/38	600,000	530,000	8:00 A.M. 10 c.c. spleen extract intravenously
	450,000	430,000	2:00 P.M.
5/11/38	550,000	530,000	8:30 A.M.
<i>Rabbit No. 1. Myelogenous Leucemia</i>			
5/24/38	420,000	460,000	3:00 P.M.
5/25/38	420,000	450,000	10:00 A.M. 10 c.c. spleen extract intravenously
5/25/38	450,000	470,000	4:00 P.M.
5/26/38	480,000	490,000	10:00 A.M.
5/27/38	490,000	440,000	10:00 A.M.

## COMMENTS

In the splenic extract from the first patient with thrombocytopenic purpura the extract in the rabbit produced an increase in the blood platelet count, while the spleen from the second patient produced a definite fall in the platelet count. Variations in both, however, were not striking and have been observed in the platelet counts of rabbits injected with a variety of materials. The divergence of these findings from those of Troland and Lee may be due to some difference in the method of extraction. In their first report they

stated that the supernatant fluid was poured off "after about two weeks," while the second report describes the periods as varying from six days to one hundred and two days.

Additional data would seem to be necessary before accepting as established, the presence of a platelet-reducing substance in the spleen of thrombocytopenic purpura.

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### THE EFFECT OF GASTRIC MUCIN ON THE HEMOGLOBIN REGENERATION IN ANEMIC DOGS\*

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THE work presented here was performed in an attempt to determine whether a commercial gastric mucin preparation stimulates blood formation in anemic dogs, through either the action of the mucin or some substance contained in the preparation. The work was prompted by the clinical observation that gastric mucin appeared to stimulate hemoglobin formation in "peptic" ulcer patients recovering from acute massive hemorrhage.

Whipple and his associates<sup>1</sup> in 1925 described a quantitative method for the determination of the ability of various substances to stimulate blood regeneration. During a period of more than a decade they have applied this method to innumerable substances.<sup>2-10</sup> Dragstedt and his co-workers<sup>11, 12</sup> have used it in an investigation of hemoglobin production in gastrectomized dogs. Riecker<sup>13</sup> has commented unfavorably on the errors of the method and the results obtained by Whipple. It was employed in the present study as the only available method for estimating "quantitatively" the ability of substances to promote hemoglobin formation.

#### METHOD

Eight dogs were used in this study. The procedure employed was essentially that of Whipple and his associates,<sup>14</sup> with slight modifications to be described below. The basal diet consisted of salmon bread to which were added the various supplements whose activities were to be determined. By means of frequent bleedings the dogs were maintained at a hemoglobin level of 5.50 to 6.50 gm. of hemoglobin per 100 c.c. of blood. When the animals

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had been satisfactorily depleted of their stores of blood-forming materials as shown by a more or less constant rate of hemoglobin formation, control periods were established, ranging from four to seven weeks.

*Series I:* Following the control periods, 25 gm. of mucin suspended in water were added to the daily basal diet for a period of two weeks, followed by a two-week period during which they received only salmon bread. Since the mucin preparation was found to contain 1 mg. of iron per gram of mucin, the dogs were then given ferric ammonium citrate equivalent to 25 mg. of iron per day for a two-week period, followed by another two-week period during which the dogs received only salmon bread.

*Series II:* This group of 4 dogs was used to investigate the effect of feeding periods of longer duration. Following the control periods, 30 gm. of mucin suspended in water were added to the basal diet daily for four weeks. The animals were then fed only salmon bread for a period of two weeks. This was followed by a four-week period during which the animals received daily by stomach tube 30 c.c. of a 0.6 per cent ferric ammonium citrate solution, an amount of iron equivalent to the iron content of the mucin previously fed. A three-week "carry-over period" on salmon bread was allowed before the next supplement was added to the diet. During the next supplemental period the dogs received daily for four weeks 250 gm. of fresh beef liver ground together with 340 gm. of salmon bread. This was followed by a four-week "carry-over period."

#### CALCULATION

Blood studies and blood volume determinations were made in essentially the same manner as that of Whipple and his collaborators. However, the amount of hemoglobin produced was calculated by a method different from that used by Whipple, as well as by the Whipple method.

The calculation of the amount of hemoglobin formed during a given period requires the consideration of two factors: (a) the amount of hemoglobin withdrawn by bleeding during the period; (b) changes in the total quantity of hemoglobin in the body during this period. The first is measured directly; the second must be calculated. Whipple's method for calculation of the hemoglobin formed may be represented by the following formula:

$$\text{Hb formed} = \text{Hb withdrawn} \div (\text{per cent Hb}_2 - \text{per cent Hb}_1),$$

where  $\text{Hb}_1$  and  $\text{Hb}_2$  are the blood hemoglobin percentages at the beginning and at the end of the period, respectively. This method arbitrarily assumes that each unit change in percentage of blood hemoglobin represents a change of 1 gm. in the total circulating hemoglobin. The assumption is valid only when the blood volume remains constant at 725 c.c. (assuming 100 per cent hemoglobin = 13.8 gm. per 100 c.c.).

The method of calculation devised by us may be represented by the following formula:

$$\text{Hb formed} = \text{Hb withdrawn} \div (\text{Hb}_2 - \text{Hb}_1),$$

where  $\text{Hb}_1$  and  $\text{Hb}_2$  represent the total circulating hemoglobin in grams at the beginning and end of the period, respectively. The accuracy of Whipple's

TABLE I  
PARTIAL PROTOCOL SERIES II

Dog No. 20

Key to Columns															
1. Days anemic 2. Weight in kilograms 3. Millions R. B. C. per c.mm. 4. Grams hemoglobin per 100 c.c. 5. Hematocrit mm. 6. Amount of blood withdrawn, c.c. 7. Centrifuge tube readings—control 8. Centrifuge tube readings—dye															
9. Reading on colorimeter 10. Plasma volume 11. Blood volume 12. Total circulating hemoglobin 13. Amount of hemoglobin withdrawn, gm. 14. Total amount of hemoglobin withdrawn, gm. 15. Correction 16. Hemoglobin produced															
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
62	12.8	4.27	6.00	20.0	70	2.1/10.7	1.5/ 8.8	0.777	789	1012	62.4	4.56	20.58	-23 10	-2.52
65			6.53		105							5.17			
67			6.26		50							3.13			
69	12.5	3.48	5.38	19.0	20	2.1/11.5	1.9/11.2	0.790	795	1022	55.0	1.08	12.86	- 7.1	5.46
73			6.41												
74			5.74												
76	12.5	4.27	5.65	19.0	16	1.9/ 9.6	1.9/ 9.6	0.770	768	1023	57.8	0.90	1.08	+ 2.8	3.88
80			5.85												
83	12.7	4.73	6.83	20.0	17	2.1/11.0	2.2/11.0	0.750	823	1087	74.2	1.16	0.90	+16.4	17.30
86			6.49		55							3.37			



TABLE II  
AVERAGE HEMOGLOBIN PRODUCTION PER WEEK

ANIMAL	B.H.P.*	MUCIN		IRON		LIVER	
		FED	CARRY- OVER	FED	CARRY- OVER	FED	CARRY- OVER
<i>A. Our Method of Calculation</i>							
Series I† 1	5.83	18.02	8.00	9.62	9.86		
Series I 2	6.53	24.97	9.91	14.63	4.84		
Series I 3	14.35	24.97	6.66	23.70	8.90		
Series I 4	15.09	21.79	15.53	15.83	6.35		
Series I Avg.	10.46±1.68	22.4±1.10	10.03	15.45±1.98	7.49		
Series II† 12	3.33	2.51	8.09	5.52	5.37	11.95	9.98
Series II 17	4.22	10.42	6.43	5.73	6.59	13.74	14.72
Series II 18	6.88	12.88	8.38	10.92	5.76	21.04	15.49
Series II 20	8.21	20.31	8.20	7.67	5.69	8.92	17.55
Series II Avg.	5.66±0.76	11.53±2.47	7.77	7.46±0.85	5.85	13.91±1.74	14.43
Average Series I and II	8.06±1.05	16.99±1.87	8.90	11.70±1.47	6.67		
<i>B. Whipple's Method of Calculation</i>							
Series I† 1	12.53	13.57	8.20	12.72	5.71		
Series I 2	8.54	26.60	10.24	13.33	6.54		
Series I 3	13.65	30.27	7.81	22.40	8.65		
Series I 4	15.75	23.79	15.68	16.64	5.30		
Series I Avg.	12.62±1.02	23.56±2.91	10.48	16.27±1.50	6.55		
Series II† 12	2.80	4.91	5.69	5.98	4.20	9.58	10.78
Series II 17	4.49	6.70	3.98	9.83	6.93	9.37	14.07
Series II 18	5.06	11.83	5.53	10.17	7.26	17.72	16.69
Series II 20	7.64	22.78	4.70	7.50	7.26	7.63	17.35
Series II Avg.	5.00±0.68	11.56±2.72	4.98	8.37±0.67	6.41	11.08±1.52	14.72
Average Series I and II	8.80±1.41	17.55±2.87	7.72	12.32±1.26	6.48		

\*Basal hemoglobin production.

†For details see Methods.

method is limited by the introduction of an arbitrary constant; the accuracy of our method is limited by the accuracy of the blood volume determinations.

## RESULTS

Table I shows a portion of a typical protocol of one of the animals used in this study. Table II shows the average weekly hemoglobin production of each of the animals during each of the experimental periods, together with the averages and probable errors. Our method was used to calculate Table IIA. Whipple's method was used to calculate Table IIB. Table III is a condensed protocol of one of the animals of the series.

The average basal hemoglobin production for all of the dogs when calculated by the Whipple method was  $8.80 \pm 1.41$  gm. per week. The addition of mucin to the diet increased the hemoglobin production to  $17.55 \pm 2.87$  gm. per week, or an increase of nearly 100 per cent, which, however, is not significant statistically. The addition of iron increased the hemoglobin production by 40 per cent. In each case the values are not statistically significant.

When our method (use of the blood volume determination) of calculation is employed, practically the same average values are obtained, but indi-

TABLE III  
CONDENSED PROTOCOL  
Dog No. 20

DAYS ANEMIC	PERIOD	WEIGHT	HB.	BLOOD VOLUME	TOTAL CIRCULATING HB.	TOTAL HB. WITH-DRAWN	HB. PRODUCED PER WEEK
62	Control	12.8	6.00	1042	62.4		
68	Control	12.5	5.38	1022	55.0	12.86	5.46
76	Control	12.5	5.65	1023	57.8	1.08	3.88
83	Control	12.7	6.83	1087	74.2	0.90	17.30
97	Control	11.6	5.50	982	54.0	19.47	-0.73*
104	Control	11.6	6.53	1083	70.8	6.54	23.34
111	Mucin	10.9	6.53	1018	66.4	12.91	8.51
118	Mucin	11.3	7.45	1018	75.7	1.11	10.41
125	Mucin	11.3	7.70	853	67.2	50.56	42.06
132	Mucin	10.8	6.00	946	56.7	30.25	20.25
146	Carry-over	10.9	6.49	964	62.6	5.9	16.40*
153	Iron	11.1	6.57	1004	65.9	1.23	4.53
160	Iron	11.0	6.97	819	57.1	8.23	-0.55
174	Iron	10.4	6.87	962	66.1	17.70	26.70*
181	Carry-over	10.5	6.30	986	62.1	7.82	3.82
188	Carry-over	9.9	6.62	942	62.3	4.78	4.98
195	Carry-over	9.9	6.85	893	61.2	9.38	8.28
202	Liver	11.1	6.15	1059	65.1	1.23	5.13
209	Liver	11.8	6.40	1620	103.6	9.15	47.65
216	Liver	12.0	5.90	1120	66.1	16.10	-21.40
223	Liver	12.5	7.20	963	69.2	1.24	4.34
230	Carry-over	12.3	7.20	1111	80.0	20.05	30.85
237	Carry-over	12.2	7.31	1062	77.6	12.56	10.16
244	Carry-over	12.7	6.53	1158	75.7	27.20	25.80
251	Carry-over	12.4	7.00	1000	72.0	9.58	3.88

\*Two weeks hemoglobin production.

vidual variations are reduced to a point where the addition of the various supplements to the diet resulted in statistically significant increases in hemoglobin production, except in the case of iron.

#### DISCUSSION

Before discussing the significance of the results obtained in this study, the reliability of the method employed to increase the rate of hemoglobin production must be considered. Examination of Table II reveals a wide variation in hemoglobin production from week to week. This variation is greatest when Whipple's method of calculation is used, a fact which is revealed by the greater probable error of the averages. However, even with our method of calculation the variations are great. These variations are inherent in the method, for examination of Whipple's data reveals equally great discrepancies. It has been impossible for us to duplicate *quantitatively* Whipple's results with iron and liver; *qualitatively* his results are confirmed. One can only conclude that under the condition of the method, the rate of hemoglobin formation is not constant and uniform when the same supplements are fed. In view of this fact *the method cannot be truly quantitative, and its results are acceptable only when the data are sufficiently extensive to permit a statistical analysis.*

The results show that mucin definitely promotes the formation of hemoglobin in the dog. Since the administration of an amount of iron equivalent

to that contained in the mucin had one-half the effect of the mucin, the activity of the latter cannot be attributed solely to its iron content. Whether the additional effect is due to a more adequate protein intake, or to some specific hematopoietic agent contained in commercial mucin, we are unable to say. These findings, however, probably explain the clinical impression that patients on mucin therapy rapidly regenerate hemoglobin.

#### SUMMARY AND CONCLUSIONS

1. In each of two series of 4 anemic dogs, a commercial gastric mucin preparation stimulated hemoglobin production 55 to 70 per cent more than could be accounted for by the amount of iron present in the preparation. However, the stimulus was not as great as when beef liver was fed.

2. A modification of Whipple's method for calculation of the results in these types of experiments has been introduced and probable errors in Whipple's calculations have been pointed out.

3. Notwithstanding our modification of the Whipple method for determining the effect of various substances on hemoglobin regeneration in anemic dogs, we believe that the inherent errors of this method make it a qualitative rather than a quantitative one, unless a sufficient amount of data are obtained to permit statistical analysis.

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# THE EFFECT OF SODIUM BROMIDE ON THE NUTRITION AND THE GASTROINTESTINAL TRACT OF EPILEPTIC PATIENTS\*

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BECAUSE of the extensive and useful administration of the bromides in epilepsy, an inquiry has been made into some possible untoward action. Among the common complaints and observations attributed to bromide intoxication are skin eruptions, drowsiness, upper respiratory infections, and, at times, anorexia and weight loss.

Lately Paskind<sup>1</sup> reported on observations of a large number of extramural epileptic patients who were treated with sodium bromide over a period of many years and showed that bromides do not cause mental deterioration, another observation sometimes attributed to the use of bromides.

In the clinic for epilepsy at the Northwestern University Medical School, bromides are found to be the most efficacious drugs in the treatment of the convulsive state. By regulating the individual dosages in accordance with clinical observations and frequent blood bromide determinations (Wuth method<sup>2</sup>), very few toxic complications have resulted.

The present study concerns itself with nutrition, function of the gastrointestinal tract, and metabolism.

Bromides, when taken orally, are rapidly absorbed from the gastrointestinal tract and also secreted by it, replacing the chloride ion (Nencki and Schoumow Simanowsky,<sup>3</sup> Bolgar<sup>7</sup>). Replacement of the chloride ion occurs in all tissues, including the blood (Boshes,<sup>4</sup> Mason<sup>5</sup>). When injected, it is rapidly secreted but slowly excreted from the body. Bromides are found normally in the body tissues in very small quantities (less than a fraction of a milligram) which vary with the diet, but they are not necessary to normal body metabolism (Winnek and Smith<sup>11</sup>).

Since the bromide ion is secreted by the stomach with the chloride ion, hydrobromic acid must be present with hydrochloric acid in the stomach secretion. The effect of hydrobromic acid on peptic digestion in *in vitro* experiments was therefore studied.

To determine peptic activity of hydrobromic acid, Mett's tubes, 20 mm. long filled with coagulated egg albumen,<sup>6</sup> were placed in different mixtures as seen in Tables I and II. It can be seen that the greater the concentration of bromide in the acid, the less digestion occurred, although the peptic digestion in all experiments reached a degree which is considered normal. One could then say that hydrochloric acid alone is a faster activator of pepsin, although the retarding action of hydrobromic acid clinically should not be apparent.

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TABLE I  
EFFECT OF HYDROBROMIC ACID ON PEPTIC DIGESTION

EXPERIMENTS	PEPSIN SOLUTION	0.18% HCl	0.18% HBr	DIGESTION IN 20 MM. METT'S TUBES		PER CENT AVERAGE DIGESTION 24 HOURS
				1	2	
1	1 c.c.	15 c.c.	0	20/20	20/20	100
2	1 c.c.	0	15 c.c.	11/20	14/20	60
3	1 c.c.	7.5 c.c.	7.5 c.c.	20/20	16/20	90
4	1 c.c.	0	0	0/20	0/20	0
		15 c.c. H <sub>2</sub> O				
5	1 c.c.	10 c.c.	5 c.c.	20/20	20/20	100

TABLE II

EXPERIMENTS	PEPSIN SOLUTION	0.2% HCl N/20	0.2% HBr N/20	DIGESTION IN 20 MM. METT'S TUBES			PER CENT AVERAGE DIGESTION 17 HOURS
				1	2	3	
1	1 c.c.	15 c.c.	0	20/20	18/20	18/20	95.0
2	1 c.c.	10 c.c.	5 c.c.	19/20	11/20	13/20	71.5
3	1 c.c.	7.5 c.c.	7.5 c.c.	16/20	13/20	14/20	71.5
4	1 c.c.	5 c.c.	10 c.c.	13/20	10/20	10/20	55.0
5	1 c.c.	0	15 c.c.	7/20	7/20	8/20	36.5

Table III indicates similar in vitro experiments to study the effect of bromide salts on tryptic digestion. Sodium bromide had no retarding effect on the tryptic digestion of casein.

TABLE III  
EFFECT OF HALIDE SALTS ON TRYPTIC DIGESTION

EXPERIMENTS	CASEIN	TRYPSIN	HALIDE SALT	DIGESTION AFTER 15 MINUTES
1	10 c.c.	1.4 c.c.	None	Complete
2	10 c.c.	1.4 c.c.	1.0 c.c. 10% NaCl	Complete
3	10 c.c.	1.4 c.c.	1.0 c.c. 10% NaBr	Complete
4	10 c.c.	1.4 c.c.	0.5 c.c. 10% NaCl	Complete
			0.5 c.c. 10% NaBr	
5	10 c.c.	1.4 c.c.	2.0 c.c. 10% NaBr	Complete

Epstein<sup>8</sup> felt that in clinical experiments in three cases, he demonstrated an increased acidity and an increase in peptic digestion by bromide medication. Since hydrobromic acid is a stronger acid than hydrochloric acid, one would expect a decrease in pH, but the slight difference would not be clinically significant.

Bromides have been recommended by many workers in functional gastrointestinal disturbances, but their actual value never has been determined. Baltaceanu<sup>9</sup> reported no constant action of bromides with or without atropine on the gastric acidity.

As previously stated, from time to time epileptic patients on bromide therapy appear to lose weight. In some anorexia is a complaint. With this in mind, a group of 34 patients, who, with the exception of one, had been followed from one to six years, was studied with regard to nutrition, gastro-

intestinal complaints, weight, gastric analysis, blood bromide, urine, stool and blood examinations. Eight of these patients suffered from an organic epilepsy and 26 from the idiopathic type.

The age distribution of the patients studied was as follows: one patient was under 10 years; 7 were from 10 to 20 years; 11, from 20 to 30 years; 7, from 30 to 40 years; 6, from 40 to 50 years; and 2, from 50 to 60 years.

The results of urine, blood, and stool examinations revealed nothing abnormal. Urines occasionally showed a trace of albumin, which usually disappeared on subsequent examinations. The results of microscopic examinations showed nothing abnormal.

Blood examinations included red and white blood cell counts and differential count and hemoglobin determinations. Mild secondary anemias were occasionally discovered even before the institution of bromide therapy, but such a condition is not unusual in clinic patients who are on an inadequate diet. This condition responded well to an antianemic regime. The results of stool examinations were all within normal limits, with the exception of positive benzidine reactions; patients with these reactions were not on meat-free diets at the time.

All patients were questioned as to the presence of gastrointestinal complaints. Many were constipated and were relieved by placing the bromide in a mixture of infusion of *Adonis vernalis*. In some cases it was necessary to give mineral oil or mild laxatives with modifications of their diet. Where gastrointestinal complaints were made in a few cases, they had been present before bromide therapy. In no case did patients have gastric disturbances directly attributable to the bromide. Most of the patients with such complaints felt better on bromides. Anorexia was not an uncommon complaint, but when present was observed in those patients who became toxic or who ran a high blood bromide (of 300+ mg. per cent). In these patients, when the blood bromide level was decreased and their diet increased, there was no loss of weight.

In Table IV one can correlate length of time patients were on sodium bromide with their weight, before and after starting medication. The state of their nutrition and average blood bromide are given.

One can see the variation in weight is within normal limits. In patient F. M. the weight loss was 18 pounds over a period of three to four years. Most of this weight loss was due to an inadequate caloric diet at the beginning of treatment. In the last year his weight has been stationary. In 5 patients, W. M., C. N., F. K., E. S., and F. M., all in the adolescent age, weight increased during their growth period in spite of their continued bromide intake. There was no correlation between duration of bromide therapy and weight gain or loss. The nutritional state was good in most of the cases and fair in only a few cases. There were no cases of malnutrition due to bromides.

The average blood bromide level attained in the clinic is usually around 200 mg. per 100 c.c. blood. With this level we see very few toxic cases, contrary to the statement of many workers that a blood bromide over 150 is toxic and dangerous.

TABLE IV

YEARS ON NaBr	NUTRITION	WEIGHT			AVERAGE BLOOD BROMIDE
		BEFORE	AFTER	DIFFERENCE	
1-2					
1. P. K.	Good	195	205	+10	225
2. E. T.	Good	182	172	-10	250
3. E. A.	Good	197	197	0	225
4. K. K.	Good	127	127	0	250
5. W. T.	Good	124	116	- 8	160
6. E. E.	Good	120	124	+ 4	250
7. N. J.	Good	130	138	+ 8	225
8. L. L.	Fair	98	94	- 4	150
9. M. P.	Fair	99	99	0	210
10. B. O.	Good	81	86	+ 5	225
11. W. M.	Good	49	66	+17	175
12. W. K.	Good	145	147	+ 2	200
2-3					
1. M. H.	Good	92	90	- 2	170
2. C. K.	Good	172	167	- 5	175
3. E. C.	Good	157	139	-18	225
4. E. R.	Fair	132	128	- 4	160
5. B. H.	Good	125	127	+ 2	160
6. M. L.	Good	120	109	-11	225
7. M. M.	Good	130	135	+ 5	175
8. C. N.	Fair	108	131	+23	125
9. L. J.	Good	138	131	- 7	175
3-4					
1. V. L.	Good	134	130	- 4	180
2. F. K.	Good	96	130	+34	175
3. C. L.	Good	193	187	- 6	190
4. I. W.	Fair	118	120	+ 2	250
4-5					
1. J. K.	Good	154	149	- 5	200
2. F. M.	Good	148	130	-18	175
3. K. R.	Fair	103	103	0	250
4. C. L.	Good	153	154	+ 1	200
6-8					
1. E. S.	Fair-Good	67	95	+28	250
2. A. J.	Good	127	128	+ 1	175
3. F. F.	Good	101	150	+49	150
4. C. R.	Good	136	136	0	200

One can reiterate that, when weight loss occurs in patients on bromides, the diet has been decreased because of anorexia, and when the diet is increased or supplemented by frequent feedings, weight can be restored.

Gastric analyses were done in 20 patients (Table V). Free acid varied from 8 to 49 and total acid varied from 24 to 65 units of N/10 hydrochloric acid. There were no patients with achylia gastrica as reported by Felsen,<sup>10</sup> or high acidity as reported by Dattner.<sup>14</sup> The total halides varied between 400 and 780 mg. per cent and the bromides from 25 to 50 per cent of the total halides. There was no relationship between change in weight and results in gastric analysis.

Because bromine is one of the halogen family, one might expect it to have an action similar to iodine. The possible action of bromide on the thyroid was studied. Margolin<sup>12</sup> and Hamilton<sup>13</sup> fed bromides to guinea pigs and albino rats and produced histologic changes in the thyroid gland similar to that produced by iodine, but not to such a marked degree. With this in mind, basal metabolic rates were determined on 13 patients when their blood

TABLE V  
GASTRIC ANALYSES

	AVERAGE BLOOD BROMIDE	ACIDITY		TOTAL HALIDES	BROMIDE	PER CENT Br/HALIDES
		FREE	TOTAL			
1. P. K.	225	49.0	65.0	630	250	40
2. V. S.	180	19.0	33.0	400	200	50
3. F. K.	175	22.0	40.0			
4. J. K.	200	37.0	62.0	450	200	44
5. E. C.	225			720	300	41
6. F. M.	175	47.5	60.5			
7. K. K.	250	22.0	36.0			
8. F. F.	150			780	260	33
9. W. T.	160	17.5	27.8	560	150	27
10. E. E.	250	+		680	250	36
11. E. H.	200	18.0	24.0	780	250	32
12. B. H.	160	19.5	27.3	620	280	45
13. C. R.	200	9.4			280	
14. M. L.	225			430	215	50
15. N. J.	225	8.0	36.0	480	125	26
16. L. L.	150	30.0	44.0			
17. M. P.	210	29.5	47.0			
18. E. S.	250	24.0	49.5			
19. C. S.	200	18.0	31.0			
20. I. W.	250	22.0	32.0			

bromide was at a high level. The rates were all within normal limits. None were over +20 or less than -20. In three cases the rates were -17, -16, and -20, but these rates could not be correlated with any other hypothyroid manifestation. One patient was toxic and ran a blood bromide over 300, but her basal metabolic rate was +7.8 per cent.

Sodium bromide is safe to use over a long period of time, but patients need to be observed for its possible toxic effects. Nutrition need not be affected. Weight can be kept at normal level with an adequate caloric intake.

#### CONCLUSIONS

1. Bromides are secreted by the stomach, displacing the chloride ions as in the blood and other tissues. Hydrobromic acid in vitro slightly retards peptic digestion but not enough to produce clinical symptoms.
2. Bromides have no retarding effect on tryptic digestion in vitro.
3. A high blood bromide has no effect on nutrition. Weight loss is produced only when there is anorexia or bromide toxicosis producing an inadequate caloric intake.
4. Gastric analyses of patients on bromides showed nothing significant. The chloride ion was displaced by bromide from 25 to 50 per cent.
5. A high blood bromide level produced no abnormal change in the basal metabolic rate.

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## THE CORRELATION OF CLINICAL, ELECTROCARDIOGRAPHIC, AND CIRCULATION TIME FINDINGS IN DETERMINING THE CARDIAC STATUS IN INFECTIOUS DISEASES\*

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THE question as to how frequently the heart is damaged during the course of infectious disease and how much such damage contributes to the symptomatology has not as yet been definitely answered. That pathologic changes of an acute nature are found on post-mortem examination in individuals who die from infectious disease has repeatedly been demonstrated in the past. This is true, however, in those who die and may not be true in survivors. Furthermore, even of those who die only a comparatively small number show pathologic changes. Thus Stone<sup>1</sup> found that of 259 autopsies of patients with lobar pneumonia, bronchopneumonia, and pneumococcus sepsis, 57 per cent of the first, 66 per cent of the second, and 38 per cent of the last had normal hearts.

An attempt has been made in the past few years to answer this question by electrocardiographic means by Brow,<sup>2</sup> Chagras,<sup>3</sup> Master and Romanoff,<sup>4</sup> Master, Romanoff, and Jaffee,<sup>5</sup> Degraff, Travell, and Yager,<sup>6</sup> Arrett and Harris,<sup>7</sup> Porter and Bloom<sup>8</sup> and others. More recently, studies of the circulation time have been used for the same purpose on 75 patients with lobar pneumonia by Hitzig, King, Bullowa, and Fishberg.<sup>9</sup> A few cases in which such study was made are also mentioned by Hitzig,<sup>10</sup> and by Tarr, Oppenheimer, and Sager.<sup>11</sup>

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No correlation has, however, been made between the clinical manifestations, the electrocardiographic changes, and the circulation time, so as to attempt to arrive at a more exact estimation of the cardiac status.

This paper is an attempt at such correlation. It covers 81 patients with infectious disease on whom the circulation time was determined at the same time that electrocardiographic studies were made, and the findings were correlated with the subjective symptoms. It adds that many more cases of circulation time studies in infectious disease to the comparatively small number recorded in the literature.

#### MATERIAL AND METHODS EMPLOYED

Our series consisted of 69 patients with lobar pneumonia, 3 with meningococcus meningitis, 4 with upper respiratory infection, including one with Vincent's angina, 3 with influenza, one with ulcerative colitis, and one with empyema of the gall bladder. There were 39 females and 42 males, varying in age between 14 and 69 years. Twelve patients, all belonging to the pneumonia group, died, and 69 recovered. Only one case had come to autopsy.

The circulation time was determined by the saccharine and ether methods at the height of the disease or when symptoms were marked. In most cases where the saccharine time was normal, no ether time determination was done. Electrocardiograms were obtained at the same time as the circulation time and were repeated at intervals of one to four days. If the circulation time was abnormal in any case, a follow-up was made. Abnormalities in heart sounds (if any), the presence or absence of murmurs, cyanosis, and objective dyspnea were recorded at the same time.

Noteworthy changes in the electrocardiogram were artificially divided into first, second, and third degrees. In the first degree were placed such changes as notching or slurring of the QRS complexes in Lead I or Lead III where previously these complexes were normal: low voltage T waves in all leads and change from positive to negative T wave in Lead III. In the second degree were included low voltage QRS complexes in all leads with slight slurring or notching; isoelectric T wave in the Lead II and negative in Lead III. In the third degree were cases showing marked notching or slurring in the QRS complex with or without low voltage, and marked changes in the T wave in Leads I and II or all leads.

The limits of normality for the circulation time were set at 9 to 16 seconds for saccharine time, and 3.5 to 8 seconds for ether time. The circulation time was obtained only in 77 patients on whom 99 saccharine time and 28 ether time determinations were done. One hundred and ninety-three electrocardiograms were done on 80 patients at varying intervals. There were 4 patients on whom no circulation time but repeated electrocardiograms were obtained, and one patient on whom no electrocardiogram was obtained but circulation time was determined.

The objective clinical signs looked for were visible dyspnea, cyanosis, cardiac murmurs, or gallop rhythm, and changes in the heart sounds, such as diminished intensity, splitting, accentuation, or reduplication.

## OBSERVATIONS

*The Electrocardiogram.*—Insignificant changes, such as slight variation in voltage of the QRS complex and of the T wave, and negative, isoelectric or diphasic P wave in Lead III, were frequently observed, but only 15 persons showed noteworthy changes that could be grouped under the first, second, and third degrees previously described. Of these the following changes were noted in the course of the disease: 2 changed from normal to first degree; 2 from normal to third degree; one from first to second; one from first to second then to normal; one from second to third degree; one from second to third degree then to normal; 4 from third degree to normal; one from third to first then to second degree; 3 from third to first degree; 2 from first degree to normal; one from second to first; and one from second to third and then first degree. All these changes occurred at various periods during the illness or in convalescence. One patient presented sinoauricular standstill with ventricular escape and ectopic auricular impulses on the third day of illness from meningitis which persisted till recovery.

The most frequent changes observed were in the direction of the T waves in Leads II and III. Only one case of prolonged P-R conduction time to 0.24 second was seen. Brow observed this condition in 14 out of 65 cases of typhoid fever. Master and his co-workers observed it in 35 per cent of cases of pneumonia. Our incidence is about the same as that of Arrett and Harris who found the condition in 2 out of 77 pneumonia patients. At no time did we observe changes in the R-T or S-T segment characteristic of early coronary occlusion reported by Master and his co-workers. An occasional slight depression or elevation and rounding was seen, but it was not characteristic of occlusion. Sometimes an auricular premature contraction was seen and sinus bradycardia and arrhythmia were frequent findings in convalescence. A few representative electrocardiograms are shown in Figs. 1 to 4.

It was found occasionally that improvement in the appearance of the T wave occurred when the clinical condition became definitely worse and even shortly before death. This is seen in Fig. 1 where all T waves became positive one day before death when they were almost isoelectric before. Post-mortem examination showed neither gross nor microscopic pathology of the heart. There was merely slight arteriosclerosis of the descending thoracic and abdominal portions of the aorta. In some cases the return to a normal T wave went hand in hand with the improvement of the patient's condition, as seen in Fig. 2.

*The Circulation Time.*—In 58 patients the saccharine time was normal and in 19 it was prolonged. Ether time was normal in 13 and prolonged in 7. Five patients showed prolonged saccharine time and normal ether time; two of these died and 3 recovered. In 3 patients the saccharine time was 8.5, 8, and 1.5 seconds, respectively, the last of which is far less than the minimum normal. Four patients had unusually prolonged saccharine time of 4, 3.5, 5 minutes, and 3 minutes 14 seconds, respectively, the first of which had also a prolonged ether time to 36 seconds. It is questionable, however, if the figures actually represent the circulation time. All 4 patients were stuporous at the time of

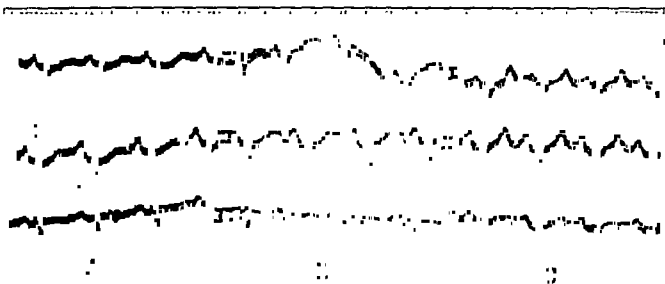


Fig. 1.—From a case of lobar pneumonia, type III, female 40 years old. A. Second day of illness. Rate 136. Tendency toward left axis deviation, slight depression of S-T segment, and very low voltage T wave in Leads I and II. Negative T wave in Lead III. B. Third day of illness. Rate 125. QRS complexes in Leads I and III are of lower voltage. C. Fifth day of illness and one day before death. Rate 136. T wave in all leads is positive.

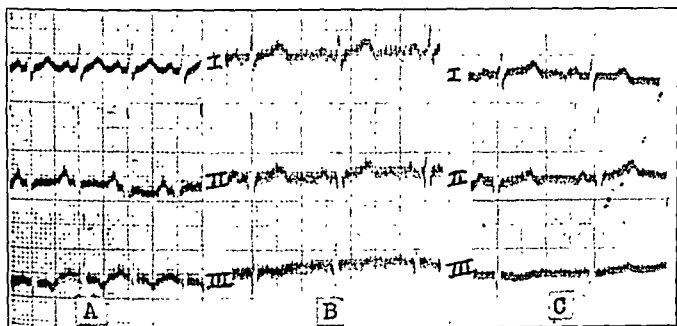


Fig. 2.—From a case of lobar pneumonia, type I, female 14 years old. A. Second day of illness. Rate 150. Isoelectric T wave in Lead II and negative T wave in Lead III. B. Fifth day of illness. Rate 85. T wave in Lead II positive and in Lead III still slightly negative. C. Eighth day of illness, beginning convalescence. Rate 83. T wave in all leads is now positive.

of the test, and it is likely that the perception apparatus was at fault. Three of these recovered and one died. The average ether time in the entire series was 7.5 seconds, and the saccharine time was 13.3 seconds. The average high saccharine time was 19 seconds.

Repeated circulation time determinations in 15 patients showed changes from time to time, as seen in Table I. Seven had a prolonged time early which returned to normal later. One of these died. In 2 patients the circulation time became faster and in 2 others it became somewhat prolonged but still within normal limits. No constant relationship was noted between the height of temperature and the rapidity of circulation time, as is shown in Table I.

The circulation time was prolonged more often among those patients who died than among those who survived, as shown in Table II. It was also found to be more frequently prolonged after 45 years of age, as shown in Table III.

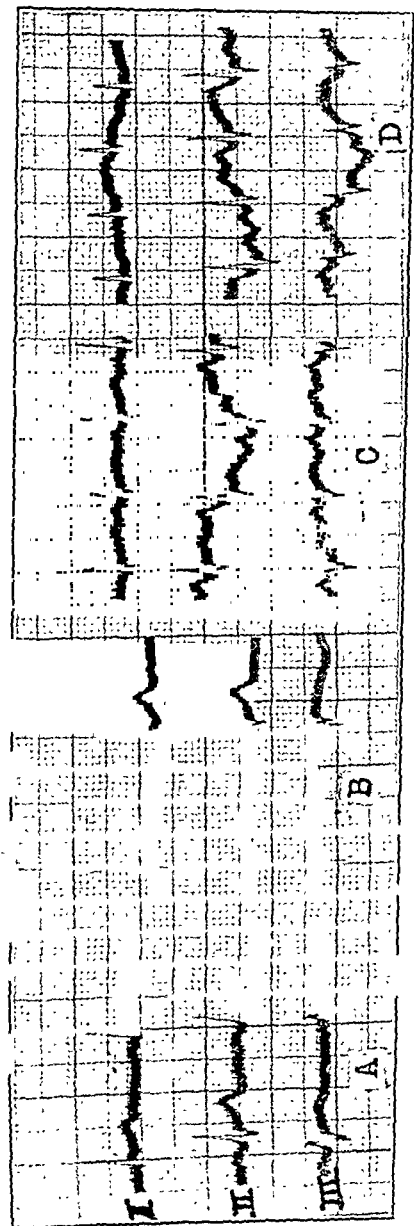


Fig. 3.—From a case of pneumonia, followed by empyema, male 30 years old. A, Third day of illness. Rate 136. Slight elevation and rounding of R-T segment in Lead I; tendency toward left axis deviation; T wave in all leads positive. B, Fifth day of illness. Rate 71. The R and T waves in Lead I of higher voltage with greater elevation and rounding of the R-T segment. C, Seventeenth day of illness. Rate 136. The QRS complex is of lower voltage and the T wave in Leads I and II approach isoelectric level, and in Lead III it is of somewhat higher voltage than previously. D, Nineteenth day of illness and one day before death. Rate 156. QRS complex in Lead I is of low voltage.

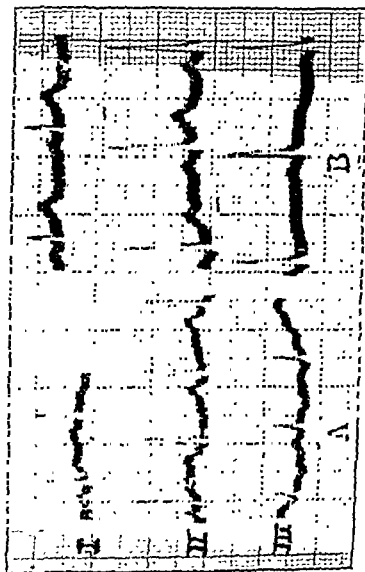


Fig. 4.—From a case of lobar pneumonia, female 26 years old. A, Third day of illness. Rate 136. Low voltage T wave in Lead II; tendency toward negative T wave in Lead II, and negative T wave in Lead III, with some complexes of higher voltage in Leads II and III. B, Tenth day after onset of illness, during convalescence. Rate 79. The QRS complex is positive in Leads I and II, and isoelectric with positive tendency in Lead III.

TABLE I  
SUMMARY OF CHANGES NOTED IN THE SACCHARINE TIME IN 15 PATIENTS

CASE NO.	DISEASE AND TERMINATION	DAY OF ILLNESS	SACCHARINE TIME	TEMPERATURE
1	Pneumonia type V Recovered	5th	11.4 sec.	104.8
		8th	9.15 sec.	104.2
2	Pneumonia type XV Recovered	4th	24.0 sec.	102.0
		5th	16.2 sec.	103.0
		12th	12.4 sec.	101.0
3	Pneumonia type I	5th	16.7 sec.	103.0
		11th	10.0 sec.	102.0
4	Pneumonia type II Recovered	6th	15.2 sec.	103.0
		10th	13.5 sec.	100.0
		12th	14.6 sec.	100.0
5	Pneumonia type VII Recovered	11th	12.2 sec.	103.4
		18th	16.2 sec.	98.6
6	Pneumonia type VI Recovered	6th	18.0 sec.	98.6
		11th	9.5 sec.	98.2
7	Pneumonia type III Recovered	7th	22.0 sec.	100.4
		8th	25.0 sec.	100.2
		17th	16.5 sec.	99.0
8	Pneumonia type I Recovered	6th	13.3 sec.	103.0
		10th	15.0 sec.	100.6
		14th	16.4 sec.	102.0
		17th	11.0 sec.	101.0
9	Pneumonia type I Recovered	2nd	20.0 sec.	106.2
		5th	22.0 sec.	99.6
		11th	12.0 sec.	98.4
		15th	13.3 sec.	98.6
10	Meningitis Recovered	3rd	18.5 sec.	102.0
		6th	12.5 sec.	98.6
11	Upper respiratory infection Recovered	2nd	19.0 sec.	100.8
		6th	19.0 sec.	98.4
12	Pneumonia type III Died	2nd	14.6 sec.	103.0
		3rd	16.1 sec.	103.0
13	Pneumonia type V Died	5th	15.2 sec.	104.0
		6th	5.0 min.	102.8
14	Pneumonia type III Died	3rd	16.5 sec.	101.4
		10th	12.2 sec.	101.2
		20th	12.2 sec.	102.0
15	Pneumonia type III Died	4th	20.0 sec.	104.4
		5th	3 min. 14 sec.	104.0

TABLE II  
RELATIVE FREQUENCY OF NORMAL AND ABNORMAL CIRCULATION TIME IN PATIENTS WHO  
RECOVERED AND THOSE WHO DIED

Only 11 patients who died had circulation time determinations done.

	PATIENTS RECOVERED		PATIENTS DIED	
	NUMBER	PER CENT	NUMBER	PER CENT
Normal Circulation Time	52	78.8	6	54.5
Prolonged Circulation Time	14	21.2	5	45.5

TABLE III  
THE RELATIVE FREQUENCY OF ABNORMAL AND NORMAL CIRCULATION TIME IN INDIVIDUALS  
ABOVE AND BELOW 45 YEARS OF AGE

	BELOW 45 YEARS		ABOVE 45 YEARS	
	NUMBER	PER CENT	NUMBER	PER CENT
Normal Circulation Time	53	80.3	5	45.5
Abnormal Circulation Time	13	19.7	6	54.5

Normality or abnormality of the circulation time did not go hand in hand with corresponding changes in the electrocardiogram, as shown in Table IV. The circulation time was as frequently prolonged when the electrocardiogram was normal as when it was abnormal. Also, marked electrocardiographic abnormalities were sometimes seen when the circulation time was normal.

TABLE IV

COMPARATIVE FREQUENCY OF NORMAL AND ABNORMAL ELECTROCARDIOGRAMS ON DAYS WHEN THE CIRCULATION TIME WAS NORMAL OR ABNORMAL

	NORMAL ELECTROCARDIOGRAM		ABNORMAL ELECTROCARDIOGRAM	
	NUMBER	PER CENT	NUMBER	PER CENT
Normal Circulation Time	34	48.6	36	51.4
Abnormal Circulation Time	14	50.0	14	50.0

In pneumonia patients the type of organism appeared to have some effect on the frequency of electrocardiographic changes and the prolongation of the circulation time, as shown in Table V. The number of cases, however, was not sufficient from which to draw definite conclusions. No relationship was noted between electrocardiographic abnormalities and clinical signs of dyspnea, cyanosis, abnormal heart sounds and murmurs, as shown in Table VI. Abnormal electrocardiograms occurred just as frequently with as without these signs. Prolonged circulation time, on the other hand, occurred more frequently in the presence of these symptoms.

TABLE V

RELATIONSHIP OF TYPE OF PNEUMONIA TO NORMAL AND ABNORMAL CIRCULATION TIME AND ELECTROCARDIOGRAM

PNEUMONIA TYPE	CIRCULATION TIME				ELECTROCARDIOGRAM			
	NORMAL		ABNORMAL		NORMAL		ABNORMAL	
	NO.	%	NO.	%	NO.	%	NO.	%
I	6	85.7	1	14.3	3	37.5	5	62.5
II	6	75.0	2	25.0	5	62.5	3	37.5
III	4	57.0	3	43.0	3	50.0	3	50.0
V	3	100.0	0		2	66.6	1	33.3
VII	6	85.7	1	14.3	5	71.0	2	29.0
VIII	0		1	100.0	1	100.0	0	
XII	1	100.0	0		0		1	100.0
XX	1	100.0	0		1	100.0	0	
XXI	1	100.0	0		0		1	100.0
Untyped	27	87.0	4	13.0	12		22	

TABLE VI

RELATIONSHIP OF OBJECTIVE SIGNS TO FREQUENCY OF NORMAL AND ABNORMAL CIRCULATION TIME AND ELECTROCARDIOGRAM

PHYSICAL SIGNS	ELECTROCARDIOGRAM				CIRCULATION RATE			
	NORMAL		ABNORMAL		NORMAL		ABNORMAL	
	NO.	%	NO.	%	NO.	%	NO.	%
Dyspnea	19	48.7	20	51.3	29	80.5	7	19.5
No dyspnea	18	48.7	19	51.3	27	84.4	5	15.6
Cyanosis	16	53.3	14	46.7	22	81.4	5	18.6
No cyanosis	18	47.4	20	52.6	28	82.5	6	17.5
Murmurs or gallop	11	55.0	9	45.0	14	73.7	5	16.3
No murmurs or gallop	30	55.5	24	44.5	41	85.4	7	14.6
Abnormal heart sounds	21	48.8	22	51.2	37	84.0	7	16.0
Normal heart sounds	19	51.3	18	48.7	31	86.0	5	14.0

## DISCUSSION

It appears from our observations that, although electrocardiographic changes frequently occur in infectious disease, they may not signify myocardial involvement. With the exception of rheumatic fever and rarely other active infections specifically involving the heart, changes in the electrocardiogram noted in the course of acute infectious disease might occur just as often in patients with or without demonstrable cardiac damage, as evidenced by clinical signs and circulation time determinations. It might be that changes are due in many cases to the febrile state itself and, in pneumonia, to such conditions as alterations in the environmental media of the heart and slight positional changes of that organ caused by solidified lung.

That fever in itself may produce changes in the electrocardiogram was demonstrated by Vesell and Bierman.<sup>12</sup> They explained the phenomenon by assuming that local cardiac and general chemical and physiologic alterations are produced by hyperthermia. Katz and his co-workers<sup>13, 14</sup> demonstrated changes in the electrocardiogram by alteration of the media adjacent to the heart. Sigler,<sup>15</sup> and previously Meek and Wilson,<sup>16</sup> Nathanson,<sup>17</sup> Kountz and Prinzmetal and others,<sup>18</sup> observed definite changes in the electrocardiogram by alteration in posture. We must, therefore, be on guard in evaluating electrocardiographic alterations in the course of acute infectious disease.

The circulation time appears to be a much better criterion of the presence of cardiac involvement in infectious disease. Our incidence of prolonged circulation time is somewhat greater than that of Hitzig, King, Bullowa, and Fishberg.<sup>9</sup> We included in the abnormal group, however, some patients who showed figures only slightly above the maximum limit of normal. The actual number of persons showing definitely prolonged circulation time would perhaps approach their figures.

It is of interest to find that prolonged circulation time occurred more frequently in patients who died than in those who survived. This would speak for cardiac failure as a contributing cause of death in a small proportion of cases. Although none of these patients presented signs of cardiac disease before, it is interesting to observe also that the circulation time is more often prolonged after 45 years of age, when degenerative changes are apt to occur.

Repeated determinations of the circulation time in the same patient revealed some variations in length from time to time. There was nearly always a tendency for it to become shorter on recovery from the disease, even if abnormally prolonged before. This would indicate that in an occasional case some degree of myocardial insufficiency exists during the course of the disease which returns to normal later.

## SUMMARY AND CONCLUSIONS

Repeated electrocardiograms and circulation time determinations were made on 81 patients with acute infectious disease, 69 of whom were patients with lobar pneumonia. Frequent changes in the electrocardiograms were noted in the course of the disease or in convalescence, some trivial, others more pronounced. The degree of changes did not go hand in hand with the severity of disease, with the degree of clinical signs, or with the length of circulation time. The



circulation time was found to be prolonged more frequently in patients who died than those who survived, and after 45 years of age than before. It was also more frequently prolonged when abnormal clinical signs were present. In an occasional case there was prolonged circulation time during the height of the disease which returned to normal later.

The findings would tend to lead to the following conclusions: 1. Circulation time is a good method to determine the cardiac efficiency during the course of acute infectious disease. 2. The electrocardiogram is of questionable value in determining the myocardial state in such disease. 3. Cardiac failure is at least partly responsible for occasional deaths in acute infectious disease. 4. It contributes at times to symptoms. 5. Latent and previously undiagnosed cardiac disease often occurs in persons past 45.

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## THE ABSENCE OF ACUTE EFFECTS OF SEX HORMONES\*

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ALTHOUGH much is known of the physiologic actions of the various estrogenic and androgenic hormones, there seem to be insufficient data to establish whether or not there are any immediate systemic effects which accompany a marked increase in circulating hormone. It has been reported<sup>1</sup> that intravenous or subcutaneous administration of crystalline menformon, although without effect in men, promptly resulted in a diminution of the blood pressure in hypertensive women, amounting to 15 to 20 mm. Hg; this substance rapidly lowered the blood pressure in female dogs but was inactive in this respect in male dogs. On the other hand, it has also been reported<sup>2-4</sup> that up to 10 R.U. of menformon or follicular extract have no effect on blood pressure or respiratory rate of dogs and cats, and that intravenous administration of quite large amounts of theelol produced no change in blood pressure, heart rate, and respiratory rate of anesthetized dogs.<sup>5</sup>

Steinach and his co-workers<sup>6</sup> have reported that testosterone and androsterone given subcutaneously reduced the blood pressure of patients after about two weeks. Greene<sup>7</sup> denies such an effect. In eunuchs, on the other hand, who are often hypotensive, testosterone seems to produce a rise in blood pressure and pulse rate.<sup>8</sup>

Since there apparently have been no studies of the immediate systemic effects of the androgens and since in the estrogen studies very small amounts have been given, we examined the effects of large doses of these hormones in 6 young adult dogs under morphine-sodium barbital anesthesia. Continuous records of two to six hours were taken of respiration by means of a pneumograph and of arterial blood pressure by a mercury manometer connected by a cannula to a carotid artery. The hormone preparations were injected directly into a cephalic vein. One female received 400 I.U. of theelin dissolved in 2 c.c. of water. One male and one female each received 50,000 I.U. of estradiol benzoate in 1 c.c. of sesame oil suspended in 2 c.c. of the animal's serum. One male and one female each received 50 mg. of testosterone propionate in 1 c.c. of sesame oil suspended in 2 c.c. of serum from the animal. The remaining animal, a male, received 45 mg. of powdered testosterone propionate suspended in 5 c.c. of the recipient's serum.

When an adequate record had been obtained, each animal was castrated, all wounds were closed, and the animal allowed to recover. After a rest period of three weeks the animal was subjected to the same procedures as

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before in an attempt to ascertain whether there is any difference in the intact and castrated animal. On both occasions the cannula for recording blood pressure was put into the left common carotid artery.

In none of the animals was there any change whatever, either before or after castration, in heart rate, blood pressure, or respiration, either immediately on the intravenous administration of the hormone or up to six hours later. The blood pressures and respirations for each animal were essentially the same in the two experiments, although in the interval between measurements the animal had undergone gonadectomy as well as recovery from the first experiment.

In order to determine whether the vasoconstrictor mechanism was capable of normal response, each animal was given a small amount of epinephrine at the end of each recording. The rise of blood pressure was in every instance quite normal.

While it is possible that such factors as removal of the hormone from the oil, hydrolysis of the hormone esters, and excretion and inactivation of the hormones by the body may have altered the effective concentration, it is, nevertheless, clear that in these experiments we have increased the amount of androgen or estrogen in the circulation as abruptly as would be possible by any mode of administration. It follows that the administration of these substances is not accompanied, at least in the dog, by immediate systemic effects.

#### SUMMARY

Intravenous administration of 50 mg. of testosterone propionate, 50,000 I.U. of estradiol benzoate, or 400 I.U. of theelin to male and female dogs, both castrated and intact, under morphine-sodium barbital anesthesia produced no change in respiration, heart rate, or blood pressure.

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## EVALUATION OF OXALATE SOLUTIONS FOR THE DETERMINATION OF PACKED CELL VOLUME IN HUMAN BLOOD\*

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**P**ACKED cell volume measurement, as a basis for the determination of the normality of an individual's blood picture, has been receiving increasing attention in recent years. The differences in the normal values reported in the literature may be attributed, in part, to the wide variation in the methods used. In the present study the Van Allen hematocrit was employed. As has been pointed out,<sup>28</sup> the accuracy of the measurement is dependent upon the amount of centrifugal force applied and on the kind and concentration of the anticoagulant used. This investigation is concerned with the latter as applied to human blood.

That the concentration of anticoagulant definitely affects the accuracy of measurement of the volume of the packed cells has been pointed out repeatedly by those who have used the method. Van Allen stated that the anticoagulant must be isotonic with the blood plasma; he also suggested the use of hirudin or heparin powder at the site of the removal of the sample, or even the use of no anticoagulant. Haden<sup>13</sup> similarly emphasized the importance of an isotonic anticoagulant.

Table I, which is a summary of anticoagulants that have been employed by other investigators, shows the wide variation in choices made, not only of kind but also of concentration.

### METHODS

In the present study the procedure employed was essentially as follows: Blood samples were taken, through finger-tip puncture, from subjects who represented chiefly women of college age (17 to 25 years), with an additional group of older women whose ages ranged from 25 to 50 years. The latter were studied for the purpose of observing whether there was associated with age a demonstrable change in the tonicity of the blood which should alter the concentration of anticoagulant to be used with older individuals.

Another point investigated was whether having the subject in basal or non-basal condition should influence the concentration of oxalate solution to be used.

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TABLE I

## KIND AND CONCENTRATION OF ANTICOAGULANTS REPORTED IN THE LITERATURE

ANTICOAGULANT	PROPORTIONS TO BLOOD	INVESTIGATOR
None		Capps <sup>4</sup>
Defibrinated blood		Bie and Möller <sup>1</sup>
Hirudin		Froehlich <sup>8</sup>
		Gram and Norgaard <sup>11</sup>
Heparin		Dieckmann and Wegner <sup>5</sup>
		Walters <sup>29</sup>
Dry sodium oxalate	100 mg. to 10 c.c. blood	Larabee <sup>14</sup>
Dry potassium oxalate	20 mg. to 10 c.c. blood	Osgood <sup>19</sup>
	10 mg. to 5 c.c. blood	Wintrobe <sup>31</sup>
	40 mg. to 10 c.c. blood	Wintrobe and Miller <sup>32</sup>
	50 mg. to 10 c.c. blood	Campbell <sup>3</sup>
Sodium oxalate (1.2%)	0.5 c.c. to 10 c.c. blood	Dulière and Adant <sup>6</sup>
Sodium oxalate (1.3%)	Van Allen hematocrit	Smith and Prest <sup>25</sup>
Sodium oxalate (1.4%)	2 c.c. to 10 c.c. blood	Haden <sup>13</sup>
Sodium oxalate (1.6%)	2 c.c. to 10 c.c. blood	Haden <sup>12</sup>
		Walters <sup>30</sup>
		Schlomowitz, Hedding, and Kehoe <sup>22</sup>
		Murphy and Fitzhugh <sup>16</sup>
Sodium citrate (3%)	0.5 c.c. to 4.5 c.c. blood	Gram <sup>10</sup>
Ammonium hydroxide (6 parts) and potassium oxalate (4 parts)	2 mg. to 1 c.c. blood	Bethell <sup>2</sup>

All subjects for whom the data are reported as being secured under basal conditions had been prepared for a basal metabolism test. For the data secured under nonbasal conditions, blood samples were obtained from the subjects without regard for consumption of food, physical activity, and time of day.

The anticoagulants selected for the present investigation included 1.6 per cent and 1.8 per cent potassium oxalate and 1.2 per cent sodium oxalate. Heparin was used as the control anticoagulant. Fresh solutions were made weekly and stored in the refrigerator. These solutions were allowed to come to room temperature before using. All oxalate solutions were tested and found to be neutral in reaction. In order to ascertain whether oven-drying of the oxalates before solutions were prepared would significantly alter the results, some samples were dried in a vacuum oven for two hours at 105° C. This procedure seemed desirable since it was planned to use the same lot of oxalate over a considerable period of time.

It was also thought advisable to validate the use of heparin as the control anticoagulant. This was accomplished by comparing the values obtained with heparin with those secured when no anticoagulant was employed. Celerity in sampling and centrifugation resulted in satisfactory determinations. A good grade of heparin was applied, dry, at the site of the puncture. The oxalate solutions under consideration were tested simultaneously, using the undried salts. All samples were prepared in duplicate and centrifuged for thirty minutes at 2750 r.p.m. in an International Centrifuge No. 2, with a head 13.5 cm. in radius. The rate of centrifugation with varying loads was checked by means of a revolution counter, and was found to yield at least 2750 r.p.m. with a maximum load.

## RESULTS

*Evaluation of Heparin as the Control Anticoagulant.*—Although it is stated quite generally<sup>15, 20, 21</sup> that heparin does not influence cell size, we were unable to locate data to support this contention.

The observed mean differences in hematocrit values of the heparinized and oxalated samples from those to which no anticoagulant was added are presented in Table II. The observations represent determinations made on from 32 to 34 different individuals, all under basal conditions. These differences are as follows: for heparin,  $-0.22$  per cent; for 1.6 per cent potassium oxalate,  $+2.70$  per cent; for 1.8 per cent potassium oxalate,  $-2.43$  per cent; and for 1.2 per cent sodium oxalate,  $+1.42$  per cent.

TABLE II

DIFFERENCES IN HEMATOCRIT VALUES OBTAINED WITH HEPARIN AND OXALATE SOLUTIONS FROM VALUES WITH NO ANTICOAGULANT

	NUMBER OF		MEAN DIFFERENCE IN PER CENT	<i>t</i>	PROBABILITY
	SUBJECTS	OBSERVATIONS			
Heparin	33	38	$-0.22$	0.05	$> 0.90$
Potassium oxalate (1.6%) (undried)	32	32	$+2.70$	3.54	$< 0.01$
Potassium oxalate (1.8%) (undried)	34	38	$-2.43$	4.91	$< 0.01$
Sodium oxalate (1.2%) (undried)	32	32	$+1.42$	3.05	$< 0.01$

The significance of these differences was determined by applying the Student "t" test<sup>27</sup> to the paired data. For the heparinized samples this difference was found to be insignificant, whereas a significant difference was observed with each of the oxalate solutions. These results justified the selection of heparin as the control anticoagulant.

TABLE III

VARIATION OF DIFFERENCES BETWEEN DUPLICATE SAMPLES

NO ANTICOAGULANT			HEPARIN			POTASSIUM OXALATE (1.6%) UNDRIED			POTASSIUM OXALATE (1.8%) UNDRIED			SODIUM OXALATE (1.2%) UNDRIED		
NO. OF DUPLICATES	MEAN OF DIFFERENCES	STANDARD DEVIATION SQUARED OF DIFFERENCES	NO. OF DUPLICATES	MEAN OF DIFFERENCES	STANDARD DEVIATION SQUARED OF DIFFERENCES	NO. OF DUPLICATES	MEAN OF DIFFERENCES	STANDARD DEVIATION SQUARED OF DIFFERENCES	NO. OF DUPLICATES	MEAN OF DIFFERENCES	STANDARD DEVIATION SQUARED OF DIFFERENCES	NO. OF DUPLICATES	MEAN OF DIFFERENCES	STANDARD DEVIATION SQUARED OF DIFFERENCES
42	0.65	0.22	71	0.60	0.24	70	0.92	0.53	70	0.79	0.35	70	1.04	0.82

A second approach to the study of the efficacy of heparin as the control consisted in evaluating the variability of the duplicates within determinations for each of the anticoagulants used. This was done by computing the mean of the differences and the standard deviation squared of the differences between duplicates. The results are recorded in Table III. It may be observed

that the variation was least for the heparinized samples and those with no anticoagulant. Of the oxalated samples the variation of those with 1.8 per cent potassium oxalate most nearly approached that of the heparinized samples, followed by those with 1.6 per cent potassium oxalate, while those with 1.2 per cent sodium oxalate showed relatively greater variation due to wide differences in three pairs of samples. These data also support the use of heparin as the control anticoagulant, despite the fact that certain investigators<sup>15, 21</sup> report difficulty with its use due to the formation of small clots which interfere with the hematocrit reading.

As a measure of the magnitude of the difference between two determinations on the same individual, the workers at Iowa State College computed the analysis of variance<sup>26</sup> on the data obtained from a series of determinations on each of 11 subjects using three anticoagulants, heparin, 1.4 and 1.6 per cent potassium oxalate. The results of these computations are given in Table IV. It is evident that there is less difference between two determinations on the same individual than there is between the means for different individuals. A highly significant difference was observed between the effects of the anticoagulants.

TABLE IV

ANALYSIS OF VARIANCE OF SEVERAL ANTICOAGULANTS (IOWA STATE COLLEGE)

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Total	74	
Methods	2	29.74*
Individuals	10	6.78*
Interaction	20	1.89
Within individuals	42	2.18

\*Highly significant.

A similar computation on a series of determinations made in this laboratory gave the values recorded in Table V. The same highly significant difference between methods was observed.

TABLE V

ANALYSIS OF VARIANCE OF SEVERAL ANTICOAGULANTS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Total	115	
Methods	3	35.33*
Individuals	13	28.80*
Interaction	39	0.56
Within individuals	60	0.92

\*Highly significant.

In the tables which follow the results obtained with oxalate solutions are expressed in terms of differences from heparin. Tables VI and VII record the data secured at the University of Minnesota and Table VIII those obtained at Iowa State College.

*Comparison of Results With Dried and Undried Salts.*—It may be observed from the mean differences in per cent between the values obtained with heparin and those with the various concentrations of oxalate employed, that oven-drying the salt resulted in all cases in solutions of greater concentration

TABLE VI  
DIFFERENCES IN HEMATOCRIT VALUES OBTAINED WITH OXALATE SOLUTIONS FROM VALUES OBTAINED WITH HEPARIN

CONDI- TION OF SUBJECT	STATE OF OXALATE	POTASSIUM OXALATE (1.6%)				POTASSIUM OXALATE (1.8%)				SODIUM OXALATE (1.2%)			
		NUMBER OF SUB- JECTS	NUMBER OF OBSER- VATIONS	MEAN DIFF- ERENCE IN PER CENT	PROBA- BILITY $t$	NUMBER OF SUB- JECTS	NUMBER OF OBSER- VATIONS	MEAN DIFF- ERENCE IN PER CENT	PROBA- BILITY $t$	NUMBER OF SUB- JECTS	NUMBER OF OBSER- VATIONS	MEAN DIFF- ERENCE IN PER CENT	PROBA- BILITY $t$
Basal	Undried	45	103	+1.53	16.78	40	55	-1.62	4.45	40	55	+2.34	6.89
Basal	Dried	24	24	+0.19	0.48	75	83	-0.85	2.70	24	24	+1.19	2.55
Nonbasal	Undried	73	84	+2.47	14.02					70	74	+2.43	6.52
Nonbasal	Dried	17	18	+2.37	3.27	10	10	-5.13	11.61	16	17	+2.17	4.25

TABLE VII  
DIFFERENCES IN HEMATOCRIT VALUES OBTAINED WITH OXALATE SOLUTIONS FROM VALUES  
WITH HEPARIN FOR THE SAME INDIVIDUALS UNDER BASAL AND NONBASAL CONDITIONS

CONDI- TION OF SUB- JECT	POTASSIUM OXALATE (1.6%)*				POTASSIUM OXALATE (1.8%)*				SODIUM OXALATE (1.2%)*			
	MEAN NO. OF DIFFER- ENCE IN SUB- JECTS	PER CENT	PROBA- BILITY $t$	PROBA- BILITY	MEAN NO. OF DIFFER- ENCE IN SUB- JECTS	PER CENT	PROBA- BILITY $t$	PROBA- BILITY	MEAN NO. OF DIFFER- ENCE IN SUB- JECTS	PER CENT	PROBA- BILITY $t$	PROBA- BILITY
Basal	35	+5.84	8.82	<0.01	33	-0.86	1.12	<0.27	33	+3.13	4.19	<0.01
Non- basal	35	+5.56	5.93	<0.01	33	+0.02	0.66	>0.18	33	+3.43	4.42	<0.01

\*Undried salts.



(Table VI). It is also apparent that all of the oxalate solutions tested, with the exception of the 1.6 per cent potassium oxalate prepared from the dried salt (subjects under basal condition), produced results which differed significantly from those obtained with heparin.

Since varying numbers of observations were made for the different solutions in this series, an additional study was undertaken to investigate more directly the effect of drying the salts upon the hematocrit values obtained. Using solutions of both the dried and undried potassium oxalate in the concentration of 1.6 per cent, duplicate samples were prepared from the same finger prick for each of 42 separate individuals. In this series the mean difference observed between the hematocrit values for the undried salt as compared with the dried was -3.78 per cent, an amount sufficiently great to be statistically significant ( $P < 0.01$ ). It is obvious that the undried salts used in the present study contained sufficient moisture to alter significantly the concentration of solutions prepared from them.

On the other hand, data secured at Iowa State College show that, when the potassium oxalate solutions were prepared from a previously unopened bottle, oven-drying the salt did not influence the results. In a series on 12 separate individuals the mean difference between the hematocrit values for heparin and those for both the dried and undried 1.6 per cent potassium oxalate solutions was 1.4 per cent. It would appear, therefore, that salts from a previously unopened bottle may not require oven-drying, whereas salts from previously opened bottles obviously require oven-drying to insure consistent results.

*Comparison of Results Under Basal and Nonbasal Conditions.*—When the mean differences from heparin for the values of the various oxalate solutions with the subjects under basal conditions were compared with the mean differences with the subjects under nonbasal conditions (Table VI), there appeared to be a trend suggestive of an increase in the tonicity of the blood under nonbasal conditions. The apparent consistency of the results in this series suggested the desirability of undertaking a further study in which direct comparisons would be made on the same individual under basal and nonbasal conditions.

The anticoagulants used in this additional series were heparin, 1.6 per cent and 1.8 per cent potassium oxalate, and 1.2 per cent sodium oxalate, prepared from the undried salts. The observed mean differences from heparin in the hematocrit values under basal and nonbasal conditions, based on 33 to 35 subjects, are presented in Table VII.

It is evident from these data that the observed differences are of essentially the same magnitude, indicating that the tonicity of the blood was unaltered.

The differences in hematocrit values with two concentrations of potassium oxalate solution from those with heparin, as observed at Iowa State College, are given in Table VIII. The determinations were made on a group of 58 women students, ranging in age from 18 to 24 years, all of whom were under nonbasal conditions. The samples were centrifuged until maximum packing

was secured (forty or more minutes) in an instrument with a head 12.5 cm. in radius, which yielded 2464 to 2488 r.p.m. with the load used. The instrument was checked repeatedly with a revolution counter.

TABLE VIII

DIFFERENCES IN HEMATOCRIT VALUES OBTAINED WITH OXALATE SOLUTIONS FROM VALUES WITH HEPARIN (IOWA STATE COLLEGE)

NUMBER OF OBSERVATIONS	POTASSIUM OXALATE (1.4%)			POTASSIUM OXALATE (1.6%)		
	MEAN DIFFERENCE IN PER CENT	<i>t</i>	PROBABILITY	MEAN DIFFERENCE IN PER CENT	<i>t</i>	PROBABILITY
58	+6.53	10.64	< 0.01	+2.07	4.66	< 0.01

It is evident from the data presented that the values for the 1.4 and 1.6 per cent potassium oxalate solutions differed significantly from those secured with heparin. The observed mean difference in hematocrit values between those with heparin and those with the 1.4 per cent potassium oxalate was +6.53 per cent; and similarly, for the 1.6 per cent potassium oxalate, +2.07 per cent.

*Comparison of Results From Two Age Groups.*—In Table IX, a comparison is made of the results with two age groups, 17 to 25 years, and 26 to 50 years. It may be noted that with the 1.6 per cent potassium oxalate the mean difference from heparin for the younger age group was +5.49 per cent, and for the older age group, +5.47 per cent. Similarly, with the 1.8 per cent potassium oxalate,

TABLE IX

DIFFERENCES IN HEMATOCRIT VALUES OBTAINED WITH OXALATE SOLUTIONS FROM VALUES WITH HEPARIN FOR TWO AGE GROUPS\*

AGE OF SUBJECTS	POTASSIUM OXALATE (1.6%)†				POTASSIUM OXALATE (1.8%)†				SODIUM OXALATE (1.2%)†			
	NO. OF SUBJECTS	MEAN DIFFERENCE IN PER CENT	<i>t</i>	PROBABILITY	NO. OF SUBJECTS	MEAN DIFFERENCE IN PER CENT	<i>t</i>	PROBABILITY	NO. OF SUBJECTS	MEAN DIFFERENCE IN PER CENT	<i>t</i>	PROBABILITY
17-25	33	+5.49	9.80	< 0.01	30	-0.60	1.37	> 0.17	30	+2.62	4.14	< 0.01
26-50	35	+5.47	8.10	< 0.01	36	-0.75	1.60	> 0.10	36	+2.86	6.89	< 0.01

\*Subjects not in basal conditions

†Salts not dried.

which was very slightly hypertonic, the mean difference for the younger age group was -0.60 per cent, and for the older age group, -0.75 per cent. From these data it is evident that there was no difference in the tonicity of the blood of these two age groups.

#### DISCUSSION

Although the importance of the concentration of the anticoagulant employed has been emphasized by a number of workers, comparatively few reports in the literature have evaluated anticoagulant solutions in terms of their tonicity with human blood. Furthermore, there appears to be considerable difference of opinion as to the concentration of solution of the various salts which are isotonic with human blood. Graff and Clarke<sup>9</sup> reported the iso-

tonicity of a solution of potassium oxalate between a concentration of 0.9 and 1.1 per cent. Haden, in his earlier work,<sup>12</sup> used 1.6 per cent sodium oxalate, but later<sup>13</sup> stated that 1.4 per cent sodium oxalate was isotonic with human blood and comparable, in its effect on cell size, to heparin and hirudin. Magath and Hurn<sup>15</sup> reported a much lower concentration of this salt (1.1 per cent) produced the same values as heparin. Norgaard and Gram<sup>16</sup> found that a 3.0 per cent sodium citrate solution did not alter cell volume, whereas Foster and Johnson<sup>7</sup> observed a 4.9 per cent shrinkage with a 1.3 per cent solution of the same salt. Such discrepancies are difficult to explain, except as being due to failure to control adequately all the conditions of the experiment.

In the present study commonly used concentrations of oxalate solutions were evaluated in terms of heparin. In addition, those factors which might explain the divergent results reported in the literature were investigated. The effect of drying the salt prior to preparation of the solution, the effect of the condition of the subject, that is, whether basal or nonbasal, and the effect of the age of the subject, were studied. Furthermore, the use of heparin as the control anticoagulant was affirmed from the standpoint of the absence of alteration in cell size and of the consistency in the results obtained.

The data secured in this laboratory show that oven-drying the salt before solutions are prepared is essential if the bottle has been previously opened, whereas the data from the Iowa State College laboratory demonstrate that if the solution is prepared from a previously unopened bottle of the salt, preliminary drying may be unnecessary.

It is obvious that any differences observed between the solutions which were prepared from the dried and undried salts cannot represent a constant relationship, since a number of factors may influence the amount of atmospheric moisture absorbed by the salt. The magnitude of these differences is such as to emphasize the importance of consistent oven-drying of the salts before preparation of solutions.

Of the solutions prepared from the dried salts, the 1.6 per cent potassium oxalate gave values which varied the least from the results secured with heparin. The 1.4 per cent potassium oxalate (Iowa State College data) and the 1.2 per cent sodium oxalate were sufficiently hypotonic to give results which differed significantly from the heparin values. The 1.8 per cent potassium oxalate, on the other hand, was hypertonic as compared with human blood, the difference again being significant.

There have appeared in the literature from time to time studies on the effect of exercise upon the composition of the blood. Changes reported, which might alter the osmotic pressure of the blood, include increases in lactic acid, serum protein, serum sugar, red blood cells, and hemoglobin.<sup>17, 23, 24</sup> In the present study the differences from heparin for the oxalate solutions were of like magnitude for the same subjects under basal and nonbasal conditions, indicating that changes which occur do not necessitate an alteration in the concentration of the anticoagulant employed.

## SUMMARY

1. A study was undertaken for the purpose of evaluating several concentrations of oxalate solutions in terms of their tonicity with the plasma of human blood.

2. The subjects for this investigation consisted of a large group of college age women (17 to 25 years), with a smaller group of older women (26 to 50 years).

3. The use of heparin as the control anticoagulant was affirmed by a study in which the results from heparin were compared with those obtained when no anticoagulant was used. The accuracy of the results with heparin was demonstrated by the calculation of the variation between duplicate determinations.

4. Analysis of variance demonstrated a highly significant difference among the means of the packed cell volume values secured with the several oxalate solutions, and among the means of the individuals' packed cell volume values.

5. Preliminary oven-drying of the salt was shown to be essential in the preparation of the oxalate solution.

6. Of the various concentrations tested, 1.6 per cent potassium oxalate, prepared from the dried salt, was found to be most nearly isotonic with human blood. The 1.4 per cent potassium oxalate and the 1.2 per cent sodium oxalate were found to be significantly hypotonic, whereas the 1.8 per cent potassium oxalate was significantly hypertonic.

7. No differences were observed in the tonicity of the blood of subjects under basal conditions as compared with those same subjects under nonbasal conditions.

8. Within the limits of the ages studied, no differences in the tonicity of the blood were observed.

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## FREQUENCY OF SYPHILIS IN OFFICE PRACTICE

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THE blood serum of 7000 consecutive office patients receiving physical examinations was tested by one or more methods for syphilis. Of the 7000 patients 4046, or 57.8 per cent, were female and 2954, or 42.2 per cent, were male. The average age was 41.91 years. Of this number positive serum reactions were obtained in 165, or 2.35 per cent. Of these 76, or 46.1 per cent, were females and 89, or 53.9 per cent, were males. The average age of this group was 34.33 years. The percentage of positives in the total group of the females was 1.8 per cent and of the males, 3.01 per cent.

Of the 7000 patients the Wassermann test alone was run on 4257 sera from 4244 patients, of which 76 were positive from 63 patients. The Kahn test alone was run on 946 sera from 870 additional patients, of which 76 were positive on 63 patients. The sera found positive by either test were run by the other method also. Two thousand one hundred and thirteen sera from 1886 patients were run with the Laughlen antigen in a comparative series with other tests, one to four other methods being run on the same serum sample. These results are as follows:

Laughlen 2113, No. positive 320
4+—177, 3+—62, 2+—55, 1+—26; Doubtful 1
Kline 1108, No. positive 269
4+—149, 3+—51, 2+—16, 1+—23; Doubtful 1
Kahn 376, No. positive 95
4+—66, 3+—16, 2+—9, 1+—4
Wassermann 1268, No. positive 154
4+—108, 3+—10, 2+—19, 1+—17
Hinton 133, No. positive 32
4+—17, 3+—0, 2+—6, 1+—9; Doubtful 1
Eagle 86, No. positive 24
4+—15, 3+—1, 2+—7, 1+—1
Rytz 60, No. positive 14
4+—11, 3+—2, 2+—1, 1+—0
Spinal fluid 17, No. positive 5

There is no relationship between the number of positives in the different methods employed, as the positive sera were repeated more often than the negative in the comparative tests.

There were 37 disagreements in this series, 25 of which were of no importance, being a minor difference in the reading of weakly positive bloods in treated cases. The 12 other disagreements are as follows:

1. Laughlen and Kline 2+, Eagle 4+
2. Laughlen 3+, Kline and Wassermann 2+, Hinton—Doubtful

3. Laughlen 3+, Kline and Kahn Negative, Wassermann 2+
4. Laughlen and Eagle 2+, Kline and Wassermann 4+
5. Laughlen 4+, Kline 2+, Kahn—Negative
6. Laughlen 4+, Kline and Kahn 2+
7. Laughlen and Kline 3+, Kahn 2+, Wassermann—Negative
8. Laughlen and Kline 3+, Kahn 1+
9. Laughlen—Negative, Kahn—Doubtful, Wassermann (twice) 4+
10. Laughlen, Hinton, and Eagle 4+, Wassermann—Doubtful
11. Laughlen and Kahn 2+, Wassermann 4+
12. Laughlen and Kahn 1+, Wassermann 4+

Seventeen spinal fluids were run, of which 5 were positive. The Kahn and Kline each showed one more positive than the Laughlen, this being accounted for by the difference in method, the Kahn concentrating the spinal fluid and the Kline the antigen. Therefore, the Laughlen may not show a weakly positive spinal fluid.

In this series were 106 cases of pregnancy, 3 of which showed 4+ positive serum reactions.

Three hundred tests were performed on whole blood with the Laughlen antigen and two mistakes were found on rechecking with the use of serum, one false positive and one false negative. This method, therefore, was discontinued.

Two hundred tests were carried out on unheated sera with both the Laughlen and the Kline antigens, and one missed 4+ was found on rechecking. This method was considered entirely unreliable.

The method found to be satisfactory with the Laughlen antigen was the same as that used with the Kline antigen: heated serum on ringed glass slides.

It was found best to use two or more methods for each serum, and the use of either the Kline and the Laughlen or the Hinton and the Laughlen tests was very satisfactory. The Kline and Laughlen methods were used more often because of the convenience of having two tests using almost identical methods and because the result may be read immediately.

#### CONCLUSION

In an unselected series of white patients presenting themselves for physical examinations in an office practice devoted to internal medicine, 7000 routine tests showed 165 positive for syphilis, or 2.35 per cent. The Laughlen antigen used in 2108 tests on 1883 patients gave equally as good results as any of the other methods employed.

## DENTAL COOPERATION IN DIABETES MELLITUS\*

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A GROUP of 125 patients with diabetes mellitus were treated in the dental department and 252 teeth were extracted. This included single and multiple extractions as well as cyst removals. A study was made from both a diabetic standpoint as well as a dental one. The diabetic study included diet, insulin dosage, and blood sugar studies (182 in number) before and after the extractions. The dental study included dental treatment, observation of the rate of healing of these patients as compared with nondiabetic persons, and observations of postoperative sequelae when present.

When observed from a diabetic standpoint, these patients may be classified on the long-time basis of insulin dosage. It was found that there were 62 mild (from 0 to 20 units of insulin) cases treated, 16 medium (from 20 to 40 units), and 13 severe (from 40 to 65 units). Other than usual postoperative bleeding occurred in one patient in the severe range (7.7 per cent), in one patient in the medium range (6.2 per cent), and in 3 patients in the mild range (5 per cent). Infected granulation tissue occurred in one of the mild cases (1.6 per cent).

Another classification from the diabetic standpoint may be made from the blood sugar level at the moment of extraction. This was taken in 91 cases. There were 36 cases in the 100 to 150 mg. range, 35 cases in the 150 to 250 mg. range, and 20 cases between 250 and 350 mg range. Four cases of bleeding occurred in the low blood sugar range (11 per cent), none occurred in the medium range, and only one occurred in the high range (5 per cent). The case of infected granulation tissue fell into the medium range.

It was found that dental surgical intervention did not cause any significant change in the blood sugar when taken immediately following the operation. The average rise or fall did not exceed 25 per cent of the original blood milligram figure, and this is within the individual error of the micro-blood-sugar method. No extreme blood sugar rise was observed, and no patient experienced insulin shock while under treatment.

Significant from the diabetic viewpoint was the fact that the time of operation was between 10:00 and 11:30 A.M., which is one and one-half hours following insulin and breakfast. At this point, the level of the blood sugar is higher than at other times of the day but descending. This at once precludes the possibility of insulin shock and untoward rise.

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The conclusions on the diabetic observations are: 1. The percentage of bleeding bore no relation to the severity of the diabetes when calculated on the basis of insulin dosage, or to the blood sugar level at the time of operation. 2. The dental interference did not cause any significant change in the level of blood sugar when the operation was concluded within two hours after breakfast and insulin.

When observed from a dental standpoint, the first consideration is the rate of healing as compared to the normal. There were only 4 cases of bleeding in a total of 125 cases observed (3.2 per cent), which is not greater than normal. Swelling occurred in 3 cases and infected granulation in one case.

Further dental considerations include the choice of anesthetic. No untoward effects due to anesthesia were observed, except nausea in 2 cases and vomiting in one case. These untoward effects occurred with nitrous oxide-oxygen and bore no relation to insulin dose or blood sugar level (2 in the medium and 1 in the high range). No such effects occurred in any range with the three types of local anesthesia used (2 per cent procaine with no epinephrine, 2 per cent procaine with 1:25 M epinephrine, and monocaine with 1:75 M epinephrine). In a person with diabetes, vomiting and nausea is of significance since his successful diabetic treatment depends on a close balance between carbohydrate absorption and insulin action. If a disturbance of this balance can be avoided, the regular diabetic routine will not be disturbed.

The conclusions from the dental point of view are: 1. The percentage of postoperative sequelae was not higher than normal. 2. General anesthesia is contraindicated due to the fact that it is prone to cause vomiting which interferes seriously with the diabetic nutrition. 3. Of the three types of local anesthesia used no significant difference in effect was observed in the diabetic patient.

## CHRONIC COR PULMONALE OF UNUSUAL ETIOLOGY\*

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THE case of chronic cor pulmonale here reported shows primary changes in the pulmonary artery and its smaller radicles with secondary pulmonary fibrosis and right heart failure. The changes in these arteries as well as others showed both acute and chronic inflammation.

### CASE REPORT

*Clinical History.*—The patient was a 45-year-old housewife who was admitted to the Rutland State Sanatorium on Dec. 3, 1937. Her past history was irrelevant, except for an unknown illness at the age of 11. She had been diagnosed at that time as having pulmonary tuberculosis. She had had no symptoms and nothing which would suggest such a diagnosis after that illness. Her present illness began at some indefinite time during the past eight or ten years. She had a chronic cough of several years' duration productive of small quantities of sputum which was never foul. She began to have intermittent attacks of dyspnea associated with some cyanosis. During an attack which was characterized by slight intermittent fever and quite marked dyspnea, the cyanosis would become more pronounced.

At the time of her entry here a physical examination showed a fairly well developed, well nourished, white adult female. She was extremely cyanotic. There was no clubbing of the nails, skin nodules, or rash noted. The principal signs were in her lungs, with dullness at the right base and occasional râles. The heart was not enlarged, and the sounds were distinct.  $P_2$  was greater than  $A_2$ . There were no murmurs. The blood pressure was 130/70. She also had a quite marked scoliosis of the dorsal spine. The spleen and liver were not palpable. The clinical laboratory findings were all negative. Her sputa were negative. There were no tubercle bacilli or fungi found. A Hinton test was negative. The urine showed a slight trace of albumin, and the blood was normal except for leucocytosis with a differential count characteristic of an acute infection. There was no eosinophilia or polycythemia. Agglutination tests for undulant fever, paratyphoid A and B, and typhoid were negative. Three blood cultures were also negative. An electrocardiogram taken two months before admission showed slight right axis deviation. An x-ray taken Jan. 6, 1938, showed some mottling at the right apex, with obliteration of the right costophrenic angle. The left was clear, except for accentuated markings.

The clinical course was characterized by progressive dyspnea and cyanosis. She continued to run only a low-grade fever and at times had intermittent pains in her chest. Her cyanosis began to deepen and oxygen therapy was instituted. This seemed to give no relief. She also began to have auricular fibrillation which cleared soon after digitalization. Another x-ray of her chest on Feb. 5, 1938, showed an infiltrative process radiating out from the left hilum which was not present in the previous film. Mottling at the right apex and at the right base which had been present was about the same. In spite of supportive measures she died of acute respiratory insufficiency with signs of right heart failure.

*Autopsy.*—The anatomic diagnoses were acute and chronic arteritis of lungs, kidneys, ileum, gall bladder, spleen, periadrenal adipose tissue, vasa vasorum of pulmonary artery, pulmonary fibrosis, congestion of left upper lobe, bronchiectasis of right lower lobe, chronic fibrous and acute fibrinous pleuritis, bronchopneumonia, perisplenitis, and leiomyomas of uterus.

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The body was fairly well developed. There was evidence of weight loss and scoliosis of dorsal spine was present. The heart weighed 300 gm., and the right ventricular wall measured 0.6 cm. and showed definite hypertrophy. The pulmonary artery was wrinkled and looked not unlike the tree bark wrinkling seen in syphilis. The aorta and other arteries examined appeared grossly normal. The abdominal organs showed cloudy swelling. The lungs were of the greatest interest. The right side of the lung cut with a great deal of difficulty, and streaks of gray fibrous tissue were present. In the lower lobe of the right lung there was a small patch of bronchiectasis. The pleural cavity on the right was obliterated. On the left there was an apparent recent pneumonitis in the lower lobe of the left lung, while the upper lobe showed fibrosis and some congestion. The cause of this marked pulmonary fibrosis was not apparent at the gross examination. The pleura of the left side of the lung showed an acute reaction.

*Microscopic Examination.*—Microscopically the heart muscles showed a few collections of cells around blood vessels; however, these were definitely not Aschoff's bodies. The pulmonary artery showed interruption of the elastica and periarterial collection of cells about the small arteries of the vasa vasorum. These arteries showed marked narrowing. The lung showed extensive fibrosis throughout. Marked changes in the small arteries were present. There were recent areas of necrosis in the media of a few small vessels, and around these vessels there were collections of polymorphonuclear cells with a few round cells. There were no eosinophiles seen. There was evidence of an older process and obliteration of some of these small arteries with recanalization of others. Some of them were completely obliterated. In the wall of the gall bladder there was also necrosis of the media of a small artery in the typical horse-collar arrangement that one sees in periarteritis nodosa. Around this small artery there was a collection of cells typical of an acute exudate. There were similar changes in the arteries about the adrenal and ileum. In the kidney the picture was not unlike that seen in focal embolic glomerular nephritis. Small thrombi were present in glomerular tufts. Wire loop lesions, such as Baehr<sup>1</sup> described in disseminated lupus, were not present. The pathology was patchy, no large vessels were involved, and no infarctions were present.

#### DISCUSSION

Clinically the diagnosis suggested chronic cor pulmonale with an obscure etiology. Tuberculosis was thought of at first because of the mottling at the right apex. Malignancy primary in the lung was also considered. Undulant fever, typhoid, subacute bacterial endocarditis, and other more obscure disorders were considered and discarded. A consultant in chest diseases called it tuberculosis. At the time of death there was no definite diagnosis.

Chronic cor pulmonale is a condition which has been described in detail in the literature. It is of manifold etiology with common causes, such as mitral stenosis, extensive pulmonary fibrosis, and emphysema secondary to asthma, chronic bronchitis, pneumoconiosis, or some other pulmonary disease. Rare causes, such as congenital cardiac defects, organic tricuspid stenosis, sickle-cell anemia, primary arteriosclerosis, or syphilis of the pulmonary artery and its branches have been described.<sup>2-7</sup>

In this particular case the pulmonary fibrosis was secondary to an acute and chronic inflammatory process beginning in the arterial system. Acute arteritis was present with acute inflammatory changes about the vessels and in the alveoli. As these acute changes subsided on previous occasions, chronic changes in the vessels as well as the pulmonary parenchyma occurred, narrowing of the lumina of small arteries and arterioles took place with, at times.

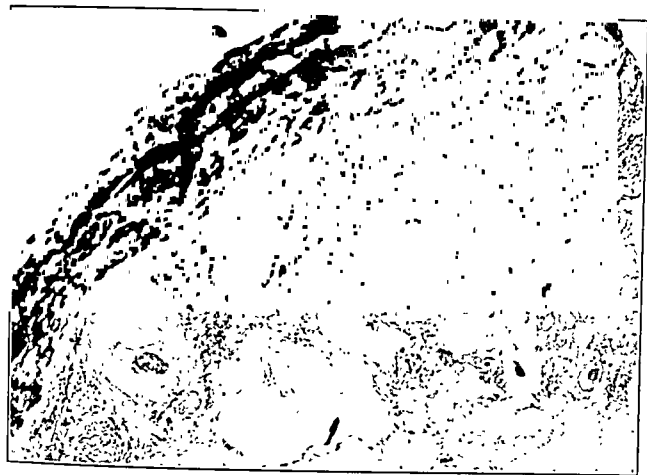


Fig. 2.

Fig. 1.—Necrotizing arteritis in small artery in wall of gall bladder. Photomicrograph  $\times 200$ .



Fig. 1.

Fig. 2.—Chronic arterial changes in lung with acute pleuritis. Photomicrograph  $\times 48$ .



Fig. 4.

Fig. 4.—Bronchiectasis. Photomicrograph  $\times 48$ .



Fig. 3.

Fig. 3.—Extensive fibrosis in lung. Chronic arteritis. Bronchiectasis. Photomicrograph  $\times 48$ .

Fig. 1.—Pulmonary artery. Note wrinkling of intimal surface and disruption of elastica. Photomicrograph  $\times 48$ .



Fig. 5.—Glomerulus showing thrombus. Photomicrograph  $\times 200$ .

obliteration and recanalization. These changes spread to the pleura and the pericardium. It is obvious that repeated attacks took place, causing more and more replacement of the lung with fibrosis. Her last attack proved too much for her cardio-respiratory system, and she died of failure of the right side of the heart. Changes in small arteries in other organs caused no impairment of function and apparently no symptoms.

Certain conditions could be definitely ruled out as a cause for this arteritis. It was not rheumatic in origin, for there was no definite history, obliteration of the lumen of the vessels does not take place in rheumatic involvement of arteries, and there were no Aschoff's bodies in the myocardium and no valvular lesions were present. It was certainly not syphilitic in origin, for there was no history of syphilis; the Hinton test was negative, the aorta was normal, and the other organs showed no evidence of syphilis.

This patient was also discussed from the viewpoint of disseminated lupus erythematosus. The clinical picture in a female with its chronicity, associated with inflammation of synovial membranes, pleura and pericardium, together with the widespread vessel involvement, suggested it. However, as Baehr and Klemperer<sup>8</sup> pointed out, the changes in the kidneys were not typical.

there was no thrombopenia, skin rash, nor endocarditis. It is of interest that in Mallory's<sup>9</sup> experience many fatal cases do not show the wire loop lesion of the kidney.

Periarteritis nodosa was strongly considered. Vessels of multiple organs were involved. There was no eosinophilia either in the circulating blood or about the vascular lesions. Infarctions and skin nodules were not present, but none of these conditions are essential. It is rather rare in the lungs. Herman<sup>10</sup> reported a single patient with rapidly progressive dyspnea, who on autopsy showed necrotizing arteritis and multiple areas of infarctions in the lung. Ophuls,<sup>11</sup> in his review, mentioned one patient who showed lung involvement. King<sup>12</sup> cited a patient in whom the x-ray and gross pathologic picture appeared like miliary tuberculosis, but who on microscopic examination showed miliary necrotizing arteritis. Gross and Friedberg,<sup>13</sup> in their discussion of periarteritis nodosa associated with rheumatic heart disease, discussed two patients who had involvement of arteries of the lungs.

Karsner<sup>14</sup> has well reviewed the situation existing today in relation to the etiology of necrotizing arteritis. The bacterial and parasitic agents known to cause necrotizing arteritis are many. Other agents, such as trypan blue,<sup>15</sup> allylamine,<sup>16</sup> injections of specific serum,<sup>17</sup> and allergic conditions<sup>18</sup> also cause this pathologic alteration in vessels. Periarteritis nodosa, a clinical entity which is not well defined because of its unknown etiologic agent, has certain criteria which some pathologists<sup>14</sup> believe noteworthy. These include the eosinophilia in the circulating blood or about the local vascular lesions and the presence of nodules formed because of the weakened vascular wall. The involvement of a large artery, the pulmonary in our case, the absence of eosinophilia either about the lesion or in the circulatory blood, the absence of nodules and infarctions, seem to rule out such a diagnosis if we accept the original description of the disease and adhere to criteria set up by Rokitsansky,<sup>19</sup> Karsner,<sup>14</sup> and other pathologists for such a diagnosis.

We are left then with a necrotizing arteritis of unknown etiology. The small patch of bronchiectasis in this case was probably secondary to the fibrosis. There is no basis for syphilis, rheumatic fever, allergic conditions, or parasitic infection. The case apparently does not fit the poorly defined syndromes of either periarteritis nodosa or disseminated lupus erythematosus. It may be due to unidentified bacteria or their products, but we have no proof of it. While the clinical and pathologic findings are of extreme interest, we must not be misled by these structural alterations. It would be much more important to know the mechanism which set these processes in motion.

#### CONCLUSIONS

1. A case of widespread, acute, and chronic arteritis of unknown etiology is reported.
2. The most marked changes occurred in the pulmonary arterial circulation with secondary pulmonary fibrosis. These changes resulted in chronic cor pulmonale.

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## AN EXPERIMENTAL STUDY OF THE RECTAL ADMINISTRATION OF MERCURIAL DIURETICS\*

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WITHIN the last few years the administration of mercurial diuretics in the form of cocoa butter suppositories has become rather widespread, and many clinical reports have appeared concerning their effectiveness and local toxicity. From a recent review of the literature<sup>1</sup> it seems evident that both of the diuretics which are available in this country as suppositories (mercurin and salyrgan) are reasonably effective. However, it has been observed that both will occasionally give rise to burning and rectal tenesmus, and proctoscopic evidence of congestion of the rectal mucosa has been reported by Crawford<sup>2</sup> and by DeGraff, Cowett, and Batterman.<sup>3</sup> Furthermore, these authors noted several instances of ulceration after the use of salyrgan but none after the use of mercurin. Although the factors which control rectal irritation are not fully known, it seemed possible that by varying slightly the composition of the salyrgan or mercurin suppository, a preparation might be obtained which would be well tolerated and still retain the character of effective diuretic with low general toxicity peculiar to organic mercury compounds of this type. It was, therefore, decided to attempt to develop an experimental procedure for the comparison of suppositories of different chemical composition, both as to absorption from the rectum and as to local toxicity, and to apply it to the study of modified forms of salyrgan and mercurin.

Suppository masses of various compositions were furnished by the manufacturers† in a concentration in cocoa butter convenient for experimental use. The salyrgan suppository has been modified in three ways, by addition (1) of theophylline; (2) of the acid of which salyrgan is the sodium salt; and (3) of both. The introduction of theophylline was suggested by pharmacologic work which demonstrated that the local toxicity is decreased<sup>4</sup> and the rate of absorption increased<sup>5</sup> by its presence. On the other hand, since mercurin consists of a mixture of acid and sodium salt and appears to be superior with regard to local toxicity, it seemed logical to determine the effect of adding salyrganic acid to salyrgan. The composition of the various suppositories used is given in Table I.

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†Salyrgan, NNR, is manufactured and distributed by Winthrop Chemical Co., Inc.; mercurin, NNR, is manufactured by Chino Chemical and Pharmaceutical Works, Ltd., and distributed by Campbell Products, Inc.

TABLE I  
COMPOSITION OF SUPPOSITORIES

CODE NO. OF EXPERIMENT- AL SUP- POSITORIES	MG. OF MERCURY PER 500 MG. DOSE (BY ANALYSIS)	RELATIVE MOLE EQUIVALENTS				
		SALYRGAN OR MERCURIN SODIUM	SALYRGANIC OR MERCURINIC ACID	SALYRGAN SODIUM THEOPHYLLINE	SALYRGANIC ACID THEOPHYLLINE	FREE THEO- PHYLLINE
		$\begin{array}{c} \text{COONa} \\ \diagup \quad \diagdown \\ \text{R}^* \end{array}$	$\begin{array}{c} \text{CO} \\ \diagup \quad \diagdown \\ \text{R} \quad \text{O.H}_2\text{O} \\ \diagdown \quad \diagup \\ \text{Hg} \end{array}$	$\begin{array}{c} \text{COONa} \\ \diagup \quad \diagdown \\ \text{R} \end{array}$	$\begin{array}{c} \text{COOH} \\ \diagup \quad \diagdown \\ \text{R} \end{array}$	$\begin{array}{c} \text{C}_7\text{H}_8\text{O}_2\text{N}_4 \end{array}$
		$\begin{array}{c} \text{HgOH} \\ \diagup \quad \diagdown \\ \text{Hg} \end{array}$		$\begin{array}{c} \text{HgC}_7\text{H}_8\text{O}_2\text{N}_4 \\ \diagup \quad \diagdown \\ \text{Hg} \end{array}$	$\begin{array}{c} \text{HgC}_7\text{H}_8\text{O}_2\text{N}_4 \\ \diagup \quad \diagdown \\ \text{Hg} \end{array}$	
Salyrgan	17.6	1.0				
618-3	20.0			1.0		
619	18.2			1.0		
ST384†	20.0			1.0		0.3
S381, 618-2	16.2	0.5	0.5			
ST382	20.0	0.3		0.2	0.5	
ST3815	20.0			0.5	0.5	
ST3825	20.0				1.0	
Mercurin	17.9	0.5	0.2			
Mercurinic acid	20.0		1.0			

\*For the structure and chemistry of salyrgan and mercurin, see Collins,<sup>6</sup> Moeller,<sup>7</sup> DeGraff,<sup>8</sup> and Lehman.<sup>9</sup>

†With sodium carbonate to a pH of 8.6.

#### PROCEDURE FOR THE COMPARISON OF TOXICITY OF SUPPOSITORIES

Cats weighing between 2 and 4 kg. were used as experimental animals. In order to standardize conditions and eliminate as much as possible the variable factor of the amount and consistency of the feces, the bowel was evacuated by oral administration of 35 c.c. of a 15 per cent solution of magnesium sulfate on two successive days. This assures soft and fairly uniform fecal material which would not be expected to interfere with the action of the suppository. The experiments were never started until forty-eight hours after the last dose of cathartic to allow any possible irritability of the bowel to subside. During this interval the animals were starved. The results of control experiments are given in the section on pathology and in Table III.

On the day of the experiment the cats were strapped to animal boards and anesthetized with 1.5 to 2.0 c.c. (0.10 to 0.13 gm.) of pentobarbital sodium intraperitoneally according to size. The suppositories were made up as small cylinders (about 6 mm. in diameter) so that 250 mg. of suppository contained 10 mg. of mercury regardless of the preparation. The doses usually given were equivalent to 10 or 20 mg. of mercury, but the concentration was always the same. This corresponds approximately to the dosage used clinically. As soon as the animals were deeply anesthetized, the suppositories were inserted by means of a glass cannula (outside diameter 1 cm.) and closely fitting plunger, pointed at one end and blunt at the other. The cannula and plunger with pointed end forward were introduced together; the shape of the end tended to overcome any spasm of the anal sphincters. The plunger was withdrawn, the suppository inserted into the cannula and pushed into place with the blunt end of the plunger.

The cannula and plunger were then carefully removed. By this procedure it was possible to place the suppositories consistently at about 6 cm. above the anal margin. Retention was 100 per cent.

The animals were killed by chloroform inhalation twenty-four hours after the insertion of the suppositories. The pelvic bone was then resected and the large intestine removed. The specimens were opened longitudinally and the feces removed under running water. Those which showed gross pathologic changes were fastened to glass frames, immersed in isotonic salt solution, and photographed on panchromatic plates. They were then fixed in Pick's solution and preserved as museum specimens. After fixing, histologic sections were made of any areas which seemed of interest.

#### PATHOLOGY

It was noted from the beginning that any of the preparations studied was capable of producing in the lower portions of the colon of the cat any of the pathologic changes to be described. However, as each series of experiments was enlarged, definite differences were found in the frequency with which lesions were produced and in the extent of involvement of the gut.

*Controls.*—The colons of 71 cats were examined as control studies for the experimental work. Of these, 55 had received no treatment related to the gastrointestinal tract, but had been sacrificed by digitalis assays. Fifty-four were entirely normal, while one exhibited a superficial yellowish exudate covering the distal 6 cm. of the bowel. This exudate was unavoidably detached during the preparation of the histologic section, and the microscopic examination revealed a normal gut wall. Five additional control cats received magnesium sulfate orally alone and 11 received the cathartic and also a 500 mg. non-medicated cocoa butter suppository. Of these 16 one showed a thin exudate similar to that just described. Only 2 of 71 control cats revealed any unusual features in the large intestine and these were of minor degree. It was thus demonstrated that the procedure involved in preliminary magnesium sulfate catharsis and the introduction of the suppository by means of the glass cannula did not produce lesions in the bowel wall. Slight degrees of congestion were seen in both control and experimental animals but these were not regarded as significant.

*Gross Examination.*—The lesions observed in the experimental animals treated with mercurial suppositories were found exclusively in the lower 12 cm. of the large bowel and could broadly be divided into two main types, exudative and necrotic. The exudative lesions varied in type from a yellow-white granular, fibrinous, superficial covering to a thick yellow or green mucoid coat which was more or less firmly attached to the gut wall (as in Fig. 1 A). In a few cases a distinct pseudomembrane was formed. The exudate covered between 2 and 12 cm. of the distal length of the gut. The necrotic lesions appeared as brownish or purplish, well-demarcated, irregular, slightly depressed areas, varying from 2 to 12 mm. in diameter (Fig. 2 A). In addition to the exudative and necrotic lesions, occasional superficial hemorrhages were noted, varying from scattered punctate lesions to larger, poorly defined areas up to 10 cm. in diameter.

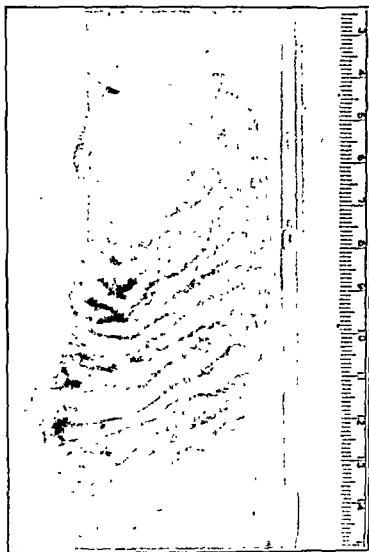


Fig. 1 A.—Gross specimen of lower half of colon of cat which had received suppository containing mercurial diuretic (Cat No. 155; dose of salyrgan equivalent to 10 mg. mercury). The exudate is extensive, yellow white, and mucoid in consistency, and is firmly attached to the gut wall. Small necrotic areas are also present but are hidden by the exudate. The pathology exhibited by this specimen was recorded as, exudate, 3 plus; necrosis, 3 plus.



ection through the specimen shown in Fig. 1 A. The mucosa is off to form part of the overlying exudate which is composed of and large mononuclear cells. There is considerable cellular in-

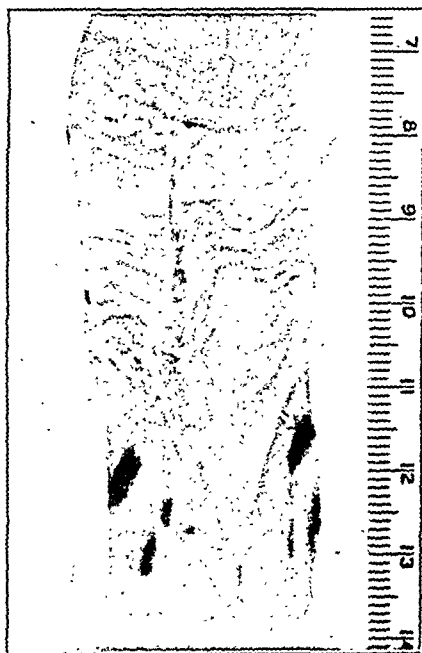


Fig. 2 A.—Gross specimen of the lower half of colon of cat which had received a suppository containing a mercurial diuretic (Cat No. 108; dose of S381 equivalent to 10 mg. mercury). The necrotic areas are 2 to 10 mm. in size, purple red, well demarcated and slightly depressed. The pathology exhibited by this specimen was recorded as, necrosis, 3 plus.

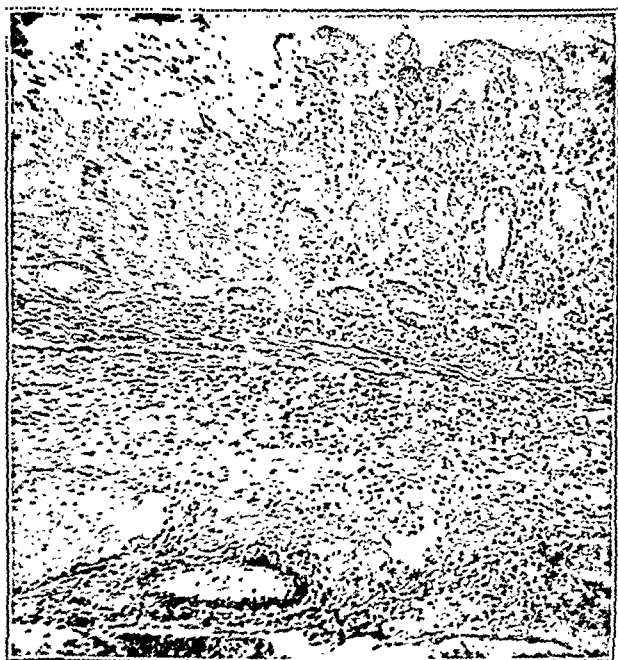


Fig. 2 B.—Microscopic section through specimen shown in Fig. 2 A. The glandular architecture can be defined, but the cells have undergone coagulation necrosis and epithelial nuclei are not visible. There is considerable cellular infiltration of both mucosa and submucosa consisting chiefly of polymorphonuclear leucocytes.

For comparison of the toxicity of the various drugs employed, the extent of the lesions was graded as one to three plus. The exudative lesions usually encircled the gut and it was therefore possible to compare them by linear measurement. Involvement of less than 2 cm. of the length of the large intestine was considered as one plus; from 2 to 6 cm., two plus; and more than 6 cm., three plus. The necrotic and hemorrhagic lesions were grouped together. A total area of involvement of less than 0.25 sq. cm. was considered one plus; from 0.25 to 1.0 sq. cm., two plus; and more than 1.0 sq. cm., three plus. On the whole, it was possible to adhere to these criteria closely, but occasionally exact measurement was difficult and required subjective judgment.

*Microscopic Examination.*—Histologic preparations were made by the paraffin block method and stained with hematoxylin and eosin. In a few cases where gross sections had shown lesser degrees of exudation, the inflammatory material was apparently dissociated from the gut wall during the making of the preparation and no alterations were visible under the microscope. In others, however, definite inflammatory exudate could be seen overlying the mucosa, consisting in varying proportions of polymorphonuclear leucocytes and large mononuclear cells embedded in amorphous pink-staining debris. In the more severe degrees of involvement the mucosa appeared necrotic and from one-third to the entire thickness was sloughed off to form part of the overlying exudate (Fig. 1 *B*). In the submucosa there was congestion of the arteries and veins and an increased cellularity consisting chiefly of mononuclear cells. In one section a small area of hemorrhagic extravasation appeared at the base of the necrotic mucosa.

Section through areas of necrosis revealed considerable alteration of the mucosa and submucosa (Fig. 2 *B*). The architecture of the mucosal glands could be defined to some degree, but the cells had undergone coagulation necrosis and nuclei were no longer visible. The glandular structure appeared to be an amorphous pinkish mass. An infiltration of polymorphonuclear cells was present throughout the mucosa and underlying submucosa and the submucosal arteries were packed with cells.

#### PROCEDURE FOR THE COMPARISON OF SUPPOSITORIES WITH RESPECT TO ABSORPTION AND MERCURY EXCRETION

For estimating absorption and excretion the animals were prepared exactly as for the study of local toxicity. However, just before administration of the suppository a catheter was inserted, fastened in place with adhesive tape, and all subsequent urine collected in a flask. The specimens were discarded if a bowel movement occurred during the experimental period. After twenty-four hours the entire large intestine was clamped at either end and excised. The twenty-four-hour urine specimens and the unopened gut were then analyzed by the method of Winkler,<sup>9</sup> modified by Gettler and Lehman.<sup>10</sup> The procedure for the digestion of the intestine and contents was the same as that described by DeGraff, Batterman, and Lehman.<sup>5</sup> Test analyses on feces and intestine by this method have not been reported and are, therefore, given in

TABLE II  
TEST ANALYSES ON LARGE INTESTINE AND CONTENTS

MG. MERCURY TAKEN	MG. MERCURY FOUND	RELATIVE PER CENT ERROR
4.99	4.57	- 8
3.49	3.27	- 6
2.49	2.47	- 1
1.47	1.31	-11
0.50	0.54	8

TABLE III  
PROTOCOLS FOR EXPERIMENTS ON THE TOXICITY OF MERCURIAL DIURETIC SUPPOSITORIES

CODE NO. OF PREPARATION	TOTAL NO. OF ANIMALS	NO. OF ANIMALS SHOWING ONE PLUS REACTION OR MORE IN EITHER CATE- GORY	NO. OF ANIMALS SHOWING EACH DEGREE OF MUCOUS OR FIBRINOUS EXUDATE	1 PLUS	2 PLUS	3 PLUS	RATIO TOTAL PLUS REACTIONS TO NO. OF AN- IMALS	NO. OF ANIMALS SHOWING EACH DEGREE OF NECROSIS OF INTESTINAL WALL	1 PLUS	2 PLUS	3 PLUS	RATIO TOTAL PLUS REACTIONS TO NO. OF ANIMALS
Controls:												
1. No treatment	54	1	-	-	1	-	-	-	-	-	-	0.11
2. Catharsis alone	11	-	-	-	1	-	-	-	-	-	-	0.06
3. Catharsis and cocoa butter suppository	9	1	-	-	-	-	-	-	-	-	-	0.25
ST3815	18	3	-	-	3	-	0.33	1	1	1	-	0.26
ST382	18	5	-	-	4	1	0.61	1	1	2	1	0.40
619	12	4	1	1	2	5	0.67	2	3	3	1	0.67
618-3	23	12	4	4	2	5	0.91	3	2	5	5	0.73
Mercurin	30	14	3	3	4	4	1.17	2	2	3	3	1.35
ST384	12	8	-	-	8	9	1.25	3	3	3	3	
Salyrgan	37	23	3	3	8	3	1.20	2	2	3	6	
S381, 618-2	20	15	1	1	7	3						

Table II. These were carried out by analyzing specimens from normal cats to which known amounts of mercuric nitrate had been added. It will be seen that the accuracy is adequate for the purposes of this study.

#### RESULTS AND CONCLUSIONS

In Table III the protocols are given for the experiments on the local toxicity of suppositories. A separate classification has been made for the two types of lesions observed since they are distinct and may be found either alone or together. For comparative purposes arbitrary numbers have been derived to indicate the relative extent and frequency of each type of reaction. The number of animals showing one-, two-, or three-plus exudative reactions was multiplied in each case by one, two, or three, and the combined total of "plus" scores in turn divided by the number of animals used. Thus, for mercurin the number 0.87 was obtained:  $[(3 \times 1) + (4 \times 2) + (5 \times 3)] \div 30$ . This process was repeated for the necrotic lesions. The ratios thus obtained were divided by the smallest of the series in either category (i.e., the ratio for compound ST3815). These numbers appear in columns 2 and 3 of Table IV. In column 4 of Table IV are listed the percentages of animals showing a one-plus reaction or greater of any type.

TABLE IV  
CORRELATION OF pH WITH RELATIVE TOXICITY OF SUPPOSITORIES

CODE NO. OF PREPARATION	RELATIVE EXTENT OF NECROSIS AND HEMORRHAGE	RELATIVE EXTENT OF EXUDATE	PER CENT OF ANIMALS SHOWING ONE-PLUS REACTION OR MORE IN EITHER CATEGORY	pH
ST3815	1	1	17	4.5
ST382	1.8	0.5	28	5.3
619	2.0	2	33	5.1
618-3	2.8	3	52	5.8
Mercurin	2.6	4	47	7.8
ST384	3.5	6	67	8.6
Salyrgan	3.8	7	62	8.9
S381, 618-2	3.6	12	75	8.6

It will be noted that of 37 cats receiving commercial salyrgan suppositories 62 per cent showed some type of reaction. The relative extent of damage to the gut wall was 3.8 times as great for salyrgan as for ST3815, the best of the preparations, and the extent of the exudative lesions was 7 times as great. Mercurin had an incidence of reactions of 47 per cent of the animals used. The extent of necrotic lesions was 2.6 times that produced by ST3815; the extent of exudative lesions, 4 times. It should be pointed out that, while the introduction of theophylline reduced the toxicity in varying degree, no preparation was found which, despite a low incidence of reactions, would not at some time cause a lesion in the colon of the cat.

Since it seemed that the pH of the medicinal constituent of the suppositories might be an important factor in determining toxicity, a portion of each suppository mass was extracted repeatedly with ether until free of cocoa butter, and the pH of a 2.5 per cent solution (or suspension) of the salt was measured accurately with the glass electrode. These values appear in column 5, Table IV.



TABLE V

MERCURY FOUND IN URINE AND LARGE INTESTINE TWENTY-FOUR HOURS AFTER ADMINISTRATION OF SUPPOSITORIES

CODE NO. OF PREPARATION	MG. Hg IN GUT	PER CENT UNABSORBED	MEAN PER CENT UNABSORBED	MG. Hg IN URINE	PER CENT EXCRETED	MEAN PER CENT EXCRETED
Mercurin	2.13	11.9	10.7	8.67	48.5	35.0
	1.20	6.7		7.79	43.5	
	-	-		7.42	41.4	
	0.67	3.7		7.30	40.8	
	1.86	10.4		7.28	40.6	
	1.33	7.4		7.00	39.1	
	4.33	24.2		3.90	21.8	
	-	-		0.74	4.1	
Mercurinic acid	11.4	57.0	40.7	3.75	18.8	15.0
	10.1	50.0		2.81	14.0	
	4.29	21.4		2.45	12.2	
ST3815	-	-	24.6	4.48	22.4	14.3
	-	-		3.86	19.3	
	1.52	7.6		3.14	15.7	
	-	-		2.76	13.8	
	-	-		2.67	13.3	
	-	-		2.37	11.8	
	5.30	26.5		2.04	10.2	
	7.93	39.6		1.57	7.8	
Salyrgan	4.56	25.9	38.9	4.34	24.6	14.2
	-	-		3.72	21.9	
	6.79	38.6		3.58	20.7	
	-	-		3.51	19.9	
	7.78	44.1		3.05	17.3	
	7.50	42.6		3.04	17.3	
	3.04	17.3		2.81	16.0	
	6.80	38.6		2.10	11.9	
	-	-		2.07	11.8	
	8.98	51.0		1.93	11.0	
	7.76	44.0		1.32	7.5	
	7.88	44.8		1.24	7.1	
	8.06	45.8		1.14	6.5	
	6.18	35.1		0.98	5.6	
ST384	-	-	10.0	7.10	35.5	10.0
	-	-		3.13	15.6	
	-	-		3.01	15.0	
	4.35	21.7		1.19	5.9	
	-	-		0.64	3.2	
	-	-		0.51	2.5	
	-	-		0.35	1.8	
	-	-		0.14	0.7	
ST3825	-	-	9.9	3.19	15.9	9.9
	-	-		2.40	12.0	
	-	-		1.33	6.7	
	-	-		0.96	4.8	
619	-	-	10.7	3.13	17.2	6.1
	-	-		1.16	6.4	
	0.32	1.8		1.07	5.9	
	2.66	14.6		1.02	5.6	
	2.28	12.5		0.67	3.7	
	0.70	3.8		0.64	3.5	
	-	-		0.58	3.2	
	3.82	21.0		0.55	3.0	
S381, 618-2	6.79	41.9	42.9	0.59	3.7	3.0
	7.16	44.2		0.53	3.3	
	6.91	42.6		0.34	2.1	

The correspondence of low pH values with lessened incidence and degree of reaction caused by various drugs is apparent. It can be seen, then, from Table IV that S381, the most toxic preparation, has nearly the highest pH, and that ST3815, the least toxic preparation, has the lowest pH. The two preparations that are available commercially, namely salyrgan and mercurin, show a definite relationship of toxicity to pH. Salyrgan with a pH of 8.9 has a toxicity almost as great as S381, whereas mercurin with a pH of 7.8 has a lower order of toxicity but is still more toxic than ST3815 with a pH of 4.5.

From the foregoing study of the local toxicity of mercurial diuretics when administered in the form of rectal suppositories, the following conclusions may be drawn:

1. All of the mercurial suppositories studied, when administered under the above-described conditions, may or may not produce pathologic changes in the large intestine of the cat, depending upon factors beyond our control at present.
2. No qualitative differences could be detected between the reactions produced by the drugs of various chemical compositions.
3. The local toxicity of mercurial suppositories may be compared by determining averages for the frequency and severity of the reactions produced in extended series of animals.
4. Apparently a distinct correlation exists between the pH of the drug in the suppository and its toxicity, preparations of low pH causing reactions least frequently. So far as local irritation is concerned, the chemical combination of theophylline with salyrgan or the admixture of salyrganic acid with salyrgan, or both, appears only to serve the purpose of reducing the pH of an aqueous solution of the suppository ingredients. It is, of course, impossible to say what theoretical connection this measured pH has with the chemical processes in the gut, but the correlation seems unmistakable.

In Table V are given the protocols for absorption from the gut and for the subsequent urinary excretion of mercury. From examination of the table the following may be concluded:

1. Absorption of mercurial diuretics, as studied by determining the mercury content of the large intestine of the cat twenty-four hours after introduction of a suppository, is highly irregular, and no correlation with local toxicity, pH, or chemical composition is apparent.
2. The excretion of mercurial diuretics, as studied by the mercury content of the urine collected during the first twenty-four hours after administration of a suppository, is also highly irregular. The values for the percentage excretion of mercury are grouped in Table V under the various preparations studied and are arranged in descending order of magnitude. In spite of the wide variations among data obtained with the same drug under conditions as nearly identical as possible, it is felt that a comparison of the means is of some value. Such a comparison shows (a) that mercurin is more rapidly excreted than any other preparation, and (b) that excretion following the administration of ST3815 is, on the average, as good as after commercial salyrgan and is better than after any of the salyrgan modifications which are of low pH and toxicity.

3. It is felt that the irregularity of absorption and excretion of mercurial diuretics after rectal administration is of significance in itself and would indicate that exact dosage by this route is impossible, even under optimum conditions.

4. It should be noted that no parallel can be drawn between the enhancing effect of theophylline upon the absorption of mercurial diuretics after intramuscular injection and its effect upon rectal absorption.

#### SUMMARY

A method for the experimental study of the rectal administration of mercurial diuretics has been suggested. A comparison has been made of the local toxicity, absorption, and elimination of suppositories of various chemical compositions. The lesions which they may produce in the colon of the cat have been described.

This investigation was carried out under the direction of Professor Arthur C. DeGraff, for whose guidance the authors wish to express their appreciation.

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# LABORATORY METHODS

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## EVALUATION OF THE TUBERCLE BACILLUS CONCENTRATION METHODS OF PETROFF, POTTENGER, AND CHEMICAL FLOCCULATION\*

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IN THE examination of sputum for tubercle bacilli many workers have realized the futility of search in a straight smear of material containing comparatively few organisms. Consequently, various methods of concentrating the bacilli have been devised. The purpose of this study was to learn what might be expected with these concentration methods in routine clinical work in an average laboratory.

The methods studied were Petroff's, Pottenger's, and chemical flocculation. The respective techniques employed are as follows:

### *Petroff's Sodium Hydroxide Method (1)*

1. Shake equal parts of sputum and 4 per cent sodium hydroxide for five minutes.
2. Incubate at 37° C. for thirty minutes or longer.
3. After complete homogenization centrifugalize at high speed.
4. Drain off supernatant fluid and neutralize sediment with normal hydrochloric acid.
5. Prepare smears from sediment, dry in air, and fix over flame.
6. Stain by Ziehl-Neelsen method.

### *Pottenger's Dilution-Flotation Method (2)*

1. Shake equal parts of sputum and 0.5 per cent sodium hydroxide for ten minutes.
2. Digest in water bath at 56° C. for thirty minutes.
3. Add 1 ml. hydrocarbon (gasoline or xylene). Add about 200 ml. distilled water and shake ten minutes.
4. Allow hydrocarbon to collect at top (fifteen to twenty minutes).
5. The hydrocarbon layer is picked up in rubber bulbed pipettes and allowed to remain in vertical position until subnatant fluid separates from hydrocarbon (five to ten minutes). Slides of desired thickness are made from hydrocarbon and allowed to dry.

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6. Wash slide in ether to remove hydrocarbon. Stain in carbolfuchsin three hours or longer. Decolorize with acid alcohol; do not allow more than thirty seconds as bacilli decolorize rapidly. If further decolorization is necessary, use 10 per cent sodium sulfate. Counterstain, preferably with 1 per cent aqueous picric acid.\*

### *Chemical Flocculation Method (3)*

1. Shake equal parts of sputum, and 4 per cent sodium hydroxide which contains 0.2 per cent  $\text{AlK}(\text{SO}_4)_2$  (alum sulfate), and 0.002 per cent bromthymol blue.

2. Digest in water bath at  $37^\circ \text{C}$ . for thirty minutes and shake for ten minutes.

3. Add N/2.5 hydrochloric acid until neutral. Shake thoroughly. If flocculation does not occur in less than five minutes, add 0.2 ml. 1 per cent  $\text{FeCl}_3$  (ferric chloride) and shake.

4. Centrifugalize at high speed to pack precipitate.

5. Prepare smears from sediment, dry in air, fix over flame, and stain by method desired.

Since it is the purpose of any concentration method to collect the greatest number of bacilli in the smallest possible volume, the first series of tests on 50 positive sputa was made to compare the number of bacilli which could be collected from 15 ml. samples of sputum sodium hydroxide digest by Petroff's method centrifugalized at 3500 r.p.m. for thirty minutes; by Pottenger's method and by chemical flocculation method centrifugalized at 1750 r.p.m. for five minutes and compared to direct smear of "choice particles" of the original sputum sample. The use of the 15 ml. sputum digest quantity was chosen for the convenient use of the 15 ml. conical centrifuge tube.

TABLE I\*

METHOD	AVERAGE NUMBER BACILLI PER FIELD	CONCENTRATION FACTOR
Straight smear of choice particles	3.3	$\times 1$
Petroff's at 3500 r.p.m.—30 min. centrifugation	19.8	$\times 6$
Pottenger's dilution-floitation	97.6	$\times 29$
Chemical flocculation at 1750 r.p.m.—5 min. centrifugation	64.2	$\times 19$

\*Mechanically homogenized. Fifty microscopic fields examined.

Following Hanks's example, direct examination was used as unity to arrive at a figure from which the concentration factor was computed. Since it is evident that the advantage of distribution of bacilli is that the chance of finding a clump of 5, 10, or 20 bacilli represents one chance, all clumps of bacilli were counted as "1"; if the clump is broken up, the chance of diagnosis is increased 5, 10, or 20 times.<sup>2</sup> As pointed out by Hanks,<sup>3</sup> the digesting process doubles the volume of sputum sample, and unless the breaking-up of clumps of bacilli is sufficient to double the possibility of finding them, the concentration factor must

\*In step 1, a 4 per cent sodium hydroxide was used in place of 0.5 per cent in order that all sputum-digest samples for the various tests may be uniform. This increase in the per cent of sodium hydroxide used necessitated the lowering of incubation temperature in step 2 to  $37^\circ \text{C}$ ., or otherwise the bacilli decolorized quite readily. Methylene blue was used as a counterstain.

be divided in two in order to indicate the actual advantage of concentration. This point was later proved as shown by Table II, assuming that hand shaking is inferior to mechanical shaking to bring about complete homogenization.

TABLE II\*

METHOD	AVERAGE NUMBER BACILLI PER FIELD HOMOGENIZED	AVERAGE NUMBER BACILLI PER FIELD OCCASIONAL SHAKING
Straight smear of choice particles	3.3 × 1'	3.3 × 1'
Petroff's at 3500 r.p.m.—30 min. centri- fugation	19.8 × 6'	14.2 × 4'
Pottenger's dilution-floitation	97.6 × 29'	47.9 × 15'
Chemical flocculation at 1750 r.p.m.— 5 min. centrifugation	54.2 × 19'	43.1 × 13'

\*Fifty microscopic fields examined.

'Concentration factor.

A second series of tests was carried out on 25 sputa which were known to be repeatedly negative by straight smear examination but positive on some previous examination. Table III shows the comparative results of the respective concentration methods.

TABLE III\*

METHOD	AVERAGE NUMBER BACILLI PER 100 FIELDS	CONCENTRATION FACTOR
Straight smear of choice particles	0.4	× 1
Petroff's at 3500 r.p.m.—30 min. centrifugation	2.9	× 7
Pottenger's dilution-floitation	13.1	× 32
Chemical flocculation at 1750 r.p.m.—5 min. centrifugation	7.4	× 18

\*Mechanically homogenized. Two hundred microscopic fields examined.

A third series of tests was performed as in the first, except that the quantity of sputum digest was not limited to 15 ml. This was undertaken to ascertain the value of the use of larger quantities of sputa. In Petroff's and the chemical flocculation methods this was found unsatisfactory, because the concentrated sediment proportionally increased in volume sometimes to the extent that only a small portion could be considered for routine use. This objection, however, was not encountered in Pottenger's method. In one instance as much as 150 ml. of the sputum digest were used without an increase in the amount of hydrocarbon necessary to collect the bacilli. Table IV shows the results of this series of tests.

TABLE IV\*

METHOD	AVERAGE NUMBER BACILLI PER FIELD†	AVERAGE NUMBER BACILLI PER FIELD‡
Straight smear of choice particles	4.1 × 1'	4.1 × 1'
Petroff's at 3500 r.p.m.—30 min. centrifugation	22.6 × 6'	23.5 × 6'
Pottenger's dilution-floitation	112.7 × 28'	163.8 × 40'
Chemical flocculation at 1750 r.p.m.— 5 min. centrifugation	64.8 × 10'	68.4 × 17'

\*25 microscopic fields examined.

†15 ml. sputum digest used.

‡Any greater volume of sputum digest used.

'Concentration factor.

It is interesting to note that in the previous series of tests the concentration factors of the respective methods remained comparatively the same, but in this series of tests the volume of sputum digest was increased; in Petroff's and the chemical flocculation methods there was a slight decrease in the concentration factor, whereas a great increase was found by the Pottenger's method. This increase of the concentration factor in Pottenger's method was persistent throughout this series of tests. On one specimen, of frothy, tenacious, mucopurulent consistency, 150 ml. of sputum digest were used. The findings were as follows: Straight smear, 50 bacilli in 50 fields, or an average of one bacilli in a field. Pottenger's concentration of the same specimen was estimated to contain 300 bacilli to a field, or a concentration factor of 300. This figure was not considered in Table IV or in the evaluation of the methods.

From the foregoing tables we derive the following concentration factors: Petroff's—sodium hydroxide method— $\times 6$ ; chemical flocculation method— $\times 18$ . The concentration factors of Petroff's and the chemical flocculation method closely resemble the findings of Hanks<sup>3</sup> in his comparison of the two methods. The concentration factor in Pottenger's method of  $\times 33$  in the series of tests with 15 ml. sputum digest volume and of  $\times 40$  in the series using greater volumes of sputum digest is considerably under  $\times 60$ , the concentration factor derived by Pottenger.<sup>2</sup> However, his method of computing the concentration factor was based on the number of bacilli found in a unit length of time, whereas the method used in this series of tests was based on the number of bacilli found per field regardless of the time consumed in the examination. It is a fact that the average time required to count the bacilli in 50 fields from preparations of straight smears, Petroff's or chemical flocculation, was as much as 200 per cent greater than the time required to count a like number of fields in preparations from Pottenger's method.

#### DISCUSSION

The criteria for judging the relative efficiency of concentration methods evaluated were based chiefly on two principles: First, the superior method must be efficient and accurate, without fail, in a reasonable length of time. Second, the technique must be simple; that is, complicated, special apparatus, unstable chemicals, and much handling must be avoided. With these points and the results of the previously related series of tests, Petroff's and the chemical flocculation methods are considered not sufficiently efficient to warrant further discussion even though their simplicity and the required length of time to perform the tests are commendable.

The superiority of Pottenger's dilution-flotation method is enhanced by its simplicity and the applicability of the method to large or small volumes of work. The apparatus which we found preferable are as follows: Phosphorus determination flask, 450 ml.—this type of flask has straight walls and a neck of 22 mm. diameter in which the hydrocarbon layer readily collects, and is sufficiently large to permit easy and thorough cleaning. Furthermore, any number of these flasks may be secured in an easily constructed wooden frame and clasped into a Kahn shaking machine for the homogenization process. The final hydrocarbon-layer separation step is carried out in Kahn serum transfer

pipettes set vertically in test tubes in a rack. Subnatant fluids from these pipettes are easily discarded and slide preparations are made of desired thickness by discharging the remainder of the bacillus-collecting hydrocarbon layer. Air - or oven-drying may be used. The slide preparations do not peel during the process of staining as so often happens in preparations from other methods. Under the microscope a maximum penetration of stain is evident. There is an absence of grit and other residues. The background is homogeneous, permitting a diffuse light. The bacilli are evenly distributed and readily recognized.

#### CONCLUSIONS

Pottenger's dilution-flotation method of concentration gives an average of 33 times as many bacilli as can be found in direct smears of choice particles in routine work. The efficiency of this method is twice that of chemical flocculation and four times that of Petroff's. According to Pottenger,<sup>2</sup> this is as accurate as guinea pig inoculation and can detect the presence of as few as 1,000 bacilli in twenty-four-hour collections of sputa, whereas to find tubercle bacilli in a straight smear by microscopic examination requires the presence of 100,000 tubercle bacilli per 1 ml. of sputum.<sup>4</sup>

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### ROUTINE LABORATORY EXAMINATIONS FOR *C. DIPHTHERIAE*\*

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ROUTINE examinations for *C. diphtheriae* have been made for so many years that one is likely to assume that this is one of the simpler bacteriologic examinations which anyone with moderate training is qualified to do, whereas in reality it is one calling for seasoned judgment and discrimination. Several years ago an error of approximately 75 per cent was found in the results obtained in one laboratory. The source of error in this case was due to placing entirely too much reliance on cellular morphology and granules. Many diphtheroids when stained for granules are indistinguishable from *C. diphtheriae*, while many strains of *C. diphtheriae* lack granules. So certain was the bacteriologist that he knew *C. diphtheriae* when he saw it that he could be convinced of his error only after careful and painstaking pure culture studies had definitely established the identity of the organism.

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Many weak points in the methods routinely used in testing for *C. diphtheriae* were recognized. During the past six or seven years a number of studies have been carried out which we believe have greatly improved the quality of our routine work.

#### LIMITATIONS OF LÖFFLER'S MEDIUM

One of the first steps in these studies was to determine the efficiency of Löffler's medium for the growth of *C. diphtheriae*. To our surprise, the quality of nearly every batch of Löffler's medium for growth and cellular morphology varied tremendously. In many instances abundant growth could be obtained, but cellular morphology was so unsatisfactory that the correct identification of various strains of *C. diphtheriae* would often be impossible. With other batches the amount of growth would be so scanty that overgrowth of *C. diphtheriae* by other throat and nose bacteria would be almost certain to occur even though in such instances cellular morphology was very satisfactory. Many months were spent in trying out different methods for the preparation of a Löffler's medium which would give reasonably good growth and satisfactory cellular morphology. The time and temperature of autoclaving and the pH of the medium were found to be the chief factors influencing growth and cellular morphology. The following method has been found to give uniformly good results.

Clear beef or hog serum	75 per cent
Beef infusion broth pH 7.6	25 per cent
Dextrose	0.25 per cent

Dissolve the dextrose in the infusion broth and add to the serum. Adjust to pH 8.3 with sodium hydroxide. Tube in 4 to 5 c.c. quantities. Place in the autoclave in a slanting position. Close the door and fasten tightly. Close the air valve to keep all air in before turning on the heat. Autoclave one hour at 15 pounds, then one and one-half hours at 20 pounds (with air retained). Final pH will be 7.6 to 7.8.

It is our practice to give every batch of Löffler's medium a number and to carefully test each for growth and cellular morphology with select strains of *C. diphtheriae*. No batch is released for routine use until it has been so tested and approved. A check in one laboratory where the Löffler's medium was found to give very poor growth but satisfactory cellular morphology revealed that this laboratory had found only about half as many positive cultures from specimens sent in for diagnosis, release, or carrier as in some others. The implication that the poor growth-promoting quality of the medium was responsible seemed justified. It was also obvious that many errors were due to failure to correctly identify *C. diphtheriae* when identification depended entirely on microscopic appearance (Table I). These limitations of Löffler's medium directed our attention to other methods.

#### ADVANTAGES OF DOUGLAS' POTASSIUM TELLURITE MEDIUM

Our interest was directed to Gilbert's modification<sup>1</sup> of Douglas' potassium tellurite medium\* because of the success others had had with this medium.

\*Dissolve 1.5 per cent of granulated agar in beef infusion broth. Adjust pH to 7.2 to 7.4, bottle in convenient amounts without filtration, and sterilize in the autoclave at 15 pounds for twenty minutes. Melt this base in an Arnold sterilizer when ready to use and cool to 50° C. Add 5.0 per cent sterile human or hog serum, 0.2 per cent of dextrose from a sterile solution (2 c.c. of a 10 per cent dextrose solution to 100 c.c. of base) and 0.01 per cent of potassium tellurite (1 c.c. of a 1 per cent sterile solution). Distribute in Petri dishes.

After using it routinely for four years and after trying McLeod's chocolate tellurite<sup>2</sup> and Claiberg's laked blood tellurite medium,<sup>3</sup> we found it the most satisfactory.\* It is easier to prepare, and all strains of *C. diphtheriae* grow well on it though colonies are much smaller than those on Claiberg's medium. It has the advantage of transparency.

The chief difficulty with this medium, as with all other media containing potassium tellurite, is controlling the concentration of tellurite. This may be accomplished by using a solution free from precipitate. We have found that potassium tellurite begins to precipitate from solution when the pH is lower than 9.0.† We, therefore, adjust the pH of a stock solution made according to the directions of Ruth Gilbert to pH 9.5 with 10 per cent potassium hydroxide. In filtering through a Seitz filter a precipitate forms in the first portion due to a change in pH by the filter pad. This portion should be refiltered by adding it to the unfiltered solution. In this way we have been able to obtain a crystal clear solution. If this is kept tightly stoppered and is withdrawn only with chemically clean pipettes, it will remain free of precipitated potassium tellurite. We have found this precipitate to be responsible for wide fluctuations in growth of *C. diphtheriae* in Douglas' medium because it varies the concentration of tellurite in the medium.

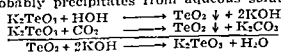
Another factor responsible for failure to find colonies of *C. diphtheriae* on tellurite plates is overgrowth by other bacteria, mostly the staphylococci. We have found that when the medium gives optimum growth of *C. diphtheriae*, overgrowth is not likely to occur since the colonies of *C. diphtheriae* may be seen protruding through the mass growth of staphylococci. When a very small number of colonies of *C. diphtheriae* are present, no doubt overgrowth does occur.

Our experience with Douglas' tellurite medium has been, briefly, that *C. diphtheriae* is frequently found when absent or unrecognized on Löffler's medium. However, when the tellurite medium is used in conjunction with Löffler's our experience has been that the examiner soon begins to recognize certain types as *C. diphtheriae* that he would not if the tellurite medium had not been used. Thus in 1934, 10.9 per cent of positive incidences were found on tellurite medium which were negative on Löffler's, while in 1935, 1936, and 1937, the percentages were much less (Table II). During 1936 many more positive results were found on Löffler's than on tellurite medium. This was due to the difficulty of obtaining the correct concentration of tellurite as indicated above. The use of both media obviated doubtful reports to physicians after 1934. The definite identification of *C. diphtheriae*, of course, is of great value in correct diagnosis by the physician and in the avoidance of unnecessary detention of cases and carriers.

The great value of tellurite plates for the accurate and dependable identification of *C. diphtheriae* is beyond question. Even with every batch of Löffler's

\*An ideal medium, however, for culturing *C. diphtheriae* from throat and nose swabs has not yet been developed. Further study of this problem is being made.

†Potassium tellurite probably precipitates from aqueous solution in the following way:



Therefore, in an excess of OH ions and with CO<sub>2</sub> ions excluded by use of a tight rubber stopper, precipitation of TeO<sub>2</sub> can be prevented. Complete solution, of course, insures a uniform concentration of potassium tellurite, and if medium and solution are carefully measured, the concentration of potassium tellurite will be constant in all batches of medium.

TABLE I

COMPARATIVE RESULTS BY DIFFERENT EXPERIENCED BACTERIOLOGISTS BASED ON EXAMINATIONS OF SMEARS FROM LÖFFLER'S MEDIUM ONLY

BACTERIOLOGIST	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
N. P. S.	-	+	-	-	-	-	-	+	-	+	-	-	-	+	-	S	+	+
O. R. W.	+	+	-	+	-	-	+	+	-	+	-	+	-	+	-	+	+	+
C. A. E.	S	+	S	-	-	-	+	+	-	+	-	-	-	+	-	+	-	-
C. A. P.	+	+	-	+	S	-	+	+	-	+	-	-	-	+	-	-	-	-
R. H. M.	S	+	S	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-
A. A. H.	+	+	-	-	-	-	+	+	S	+	-	S	-	+	+	S	+	+
M. W.	+	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	+	+
T. F. D.	+	+	+	-	-	S	+	+	S	+	-	+	-	S	-	+	+	-

S = suspicious.

Note: Preparations selected because of difficulty in decision as to whether or not they were diphtheria.

TABLE II  
COMPARISON OF NUMBER AND PER CENT OF POSITIVE CULTURES OF C. DIPHTHERIAE ON LÖFFLER'S AND DOUGLAS' MEDIA

YEAR	LÖFFLER + TELLURITE +		LÖFFLER + TELLURITE - OR UNS.		LÖFFLER - OR UNS. TELLURITE +		TOTAL POSITIVES
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	
1934*	295	80.6	9	2.5	62	17.0	366
1935†	492	91.3	25	4.6	22	4.1	539
1936*	218	80.8	32	11.8	20	7.4	270
1937†	1636	88.5	72	6.1	69	5.9	1177

\*Central laboratory.

†Central and branch laboratories.

Uns. = unsatisfactory.

medium carefully controlled, it is never possible to be certain of many results if only Löffler's medium is used. No bacteriologist in our service who has once become accustomed to the use of tellurite plates has felt that he could again depend solely on Löffler's. It is our practice to read all tellurite plates after twenty-two to twenty-four hours' incubation at 37° C. and to pick suspicious and typical colonies to microslides. These are stained with Löffler's methylene blue. It is usually a simple matter to identify the various types and strains of *C. diphtheriae* in these pure or almost pure cultures from tellurite plates after one becomes familiar with the cellular morphology of *C. diphtheriae* on this medium. We have found<sup>4</sup> that an experienced person can identify cultures of *C. diphtheriae* as *gravis*, *mitis*, or *intermediate* types with a high degree of accuracy, from cellular morphology on tellurite plates. The use of tellurite plates greatly facilitates virulence tests when these are necessary. Colonies may be picked without delay from primary plates, and there is not the chance of picking several colonies which may be descendants of one organism as when the growth from a Löffler slant is plated. Through the use of tellurite plates one is unquestionably enabled to recognize *C. diphtheriae* on Löffler's with far greater accuracy than is otherwise possible.

We find, however, a considerable percentage of Löffler slant cultures positive when tellurite plates are negative (Table II). It is our practice to examine all cultures for diagnosis or release on both media. However, where we have large numbers of throat and nose swabs from groups of school children and others for detection of carriers, we usually use either tellurite plates or Löffler slants, but not both, due to the work involved and the doubtful importance of carriers, with very few diphtheria organisms, in the spread of the disease. With proper controls tellurite plates are not believed to be negative except rarely when very small numbers of *C. diphtheriae* are present in the throat or nose. Not more than one specimen should be inoculated onto a single plate except in emergencies. Obviously, specimens from different persons should never be inoculated on one plate.

Douglas' tellurite medium is prepared as slants and is used in containers for mailing specimens to the laboratory. These slants are sealed with paraffined cork stoppers and carefully tested for sterility. The tellurite medium has been found to inhibit spore formers and spreaders, but in the strength used has not been found to prevent the growth of either *C. diphtheriae*, hemolytic streptococci, or pneumococci. Oftentimes the tellurite slants are positive for *C. diphtheriae* as received. We believe the medium is superior to Löffler slants for this purpose.

#### VALUE OF DIRECT SMEARS IN DIAGNOSIS OF DIPHTHERIA

When diphtheria bacilli can be identified by direct microscopic examination of stained preparations of material from the throat or nose lesions, this is the method of choice, for it permits an immediate report. Frequently the organisms are as distinctive in such direct smears as in cultures. At times they occur in large numbers. After several years' experience with direct smears, bacteriologists both in our central and branch laboratories have been able to find positives in an increasing percentage of cases (Table III). Cultures are always made

TABLE III  
VALUE OF DIRECT EXAMINATIONS FOR *C. DIPHTHERIAE*

	1933	1934	1935	1936	1937
Number of positive cases with satisfactory direct smears	221	175	211	170	187
Number of positive cases on direct smear	62	98	90	91	132
Per cent positive	28.0	56.0	44.8	53.5	70.7
Number of errors, direct smear positive, culture negative	4	6	6	0	1
Per cent of error	1.8	3.4	2.8	0	0.5

NOTE: Only those specimens for diagnosis on which a satisfactory direct smear was made and on which the cultures subsequently yielded *C. diphtheriae* are considered in this tabulation.

regardless of the finding of *C. diphtheriae* in direct smears. During the past two years only one direct smear was considered positive which did not confirm on culture. In many instances only scanty material was obtained on the swabs. Undoubtedly, a very large percentage would be positive in this way if physicians would use care to secure part of the membrane on the swab. By emphasis on this point we hope to increase still further the percentage of positive direct smears.

In the cases with positive direct smears the physician is given this information by telephone as a preliminary report. A written report based on culture is sent later. All preparations, whether direct smears or cultures, are stained with Löffler's methylene blue. No other stain has been found as satisfactory. On several occasions preparations have been found which were stained purplish rather than blue, and in one instance at least, a large error was the result of incorrect staining. On investigation it was found that too much alkali had been added to the stain. It is important that the final pH of the methylene blue be approximately 6.0.

#### INCIDENCE OF NASAL DIPHTHERIA

It is a common practice in taking cultures for the diagnosis, release, or detection of diphtheria carriers to take material only from the throat. While the fallacy of such a practice is obvious, a full appreciation of the matter does not always exist. One of us was a carrier of a nonvirulent strain of *C. diphtheriae* for several years and being subject to frequent attacks of sinusitis, he made repeated cultures of the discharge from the nose. The danger from any one with discharging sinuses who happened to be a carrier of a virulent strain of *C. diphtheriae* was obvious from the fact that enormous numbers of *C. diphtheriae* were frequently found during such attacks. Since the hands are perhaps more frequently soiled with discharges from the nose than from the throat, particularly during colds when such contamination may be very great, it is apparent that people with positive nose cultures are most likely to spread the disease. The danger from carriers is probably not during the time when they harbor a few virulent diphtheria bacilli, but during the time when such carriers develop a cold and virulent diphtheria organisms are present in enormous numbers. In order to have some quantitative data on the incidence of nasal infections, the percentage of positive nose cultures was compiled for three years. The percentage of cases with both throat and nose positive was 18.5, 19.0, and

26.1, respectively. The percentages of cases with positive nose and negative throat cultures were 13.8, 12.0, and 2.5. The total percentages for cases with positive throat or nose cultures were, therefore, 32.3, 31.0, and 28.6. Almost a third of all persons carrying *C. diphtheriae* carried this organism in the nose (Table IV).

TABLE IV  
INCIDENCE OF NASAL INFLECTION OF DIPHTHERIA

	1933		1934		1935	
	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
Total positives (nose and throat)	563	100.0	782	100.0	241	100.0
Nose and throat positive	105	18.5	150	19.0	63	26.1
Nose positive, throat negative	76	13.8	96	12.0	6	2.5
Nose negative, throat positive	380	67.8	536	68.5	172	71.4

#### DISCUSSION AND SUMMARY

Though it is generally accepted that Löffler's medium is the one of choice for routine culturing of *C. diphtheriae* from throat and nose swabs, the growth and cellular morphology of various strains of *C. diphtheriae* on different batches of this medium have been found so variable that errors as high as 50 to 75 per cent have occurred at times. Unless Löffler's medium is very carefully prepared with particular attention to pH, time, pressure, and air conditions during autoclaving, satisfactory batches of this medium cannot be obtained. Every batch should be tested for final pH and for growth and cellular morphology of select strains of *C. diphtheriae*. Even with such care, Löffler's medium is not entirely satisfactory, for overgrowth by other bacteria often occurs and at times examinations are impossible due to liquefaction. Even when the medium is adjusted to insure optimum growth and cellular morphology for *C. diphtheriae*, the identification of this organism on Löffler's medium alone cannot, in many instances, be made with certainty. This makes it necessary for the laboratory to give suspicious or doubtful reports which are unsatisfactory both to physicians and health officers.

Serum tellurite agar plates have been used routinely in the laboratories of the Maryland State Department of Health for five years. These have been of such value that none of the more than a dozen bacteriologists who have become familiar with the use of this medium would want to depend solely on Löffler's medium. The most important result from the use of tellurite plates has been the elimination of all suspicious or doubtful reports. Nervous strain, due to the tension of trying to decide if certain *C. diphtheriae*-like bacilli are really diphtheria bacilli or diphtheroids, is eliminated. Colonies of *C. diphtheriae* can usually be identified by a simple inspection of the plates, though colonies should always be transferred to slides for a microscopic check. The cellular morphology of *C. diphtheriae* on tellurite medium, while different from that on Löffler's medium, is very characteristic. Not only can *C. diphtheriae* be easily differentiated from the diphtheroids but with experience those interested can learn to identify the types (*gravis*, *mitis*, and *intermediate*) if necessary, with a high degree of accuracy.

The tellurite medium described has also been found to be superior (in slant form) to Löffler's medium for use in specimen mailing outfits. It is easily prepared, adheres more tenaciously to the tubes, is not easily broken up, and is more resistant to contamination. It suppresses growth of liquefying spore-formers and many other bacteria, but permits the growth of *C. diphtheriae*, hemolytic streptococci, and pneumococci.

The accuracy of the direct smear in the laboratory diagnosis of diphtheria has been carefully studied. During the last three years over half the swabs from cases of diphtheria have been positive by direct smear. During the past two years, only one direct smear reported as positive has failed to be confirmed. In many of these smears there was insufficient material for a proper examination. If the cooperation of physicians can be secured to get part of the necrotic tissue from the lesion on the swab, there is little question that positive results could be obtained in most instances. The direct smear in the hands of experienced workers is of utmost value.

The incidence of nasal infections has been found very high. Nearly one-third of all cases had nose infections. The danger from spreading diphtheria is probably greater in children and adults with nose infections than in those with the site of infection only in the throat. This danger may be paramount when the individual develops a cold. Routine cultures of the nose are just as important as cultures of the throat.

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## A NEW AND MORE PRACTICAL CULTURE TUBE DESIGNED FOR MORE RAPID DIAGNOSIS\*

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THE wise and time-honored teaching for many years current in our American medical colleges—"If in doubt, give diphtheria antitoxin!"—has been the result of the slower methods of culturing, growing, and recognizing by direct microscopic examination of the stained smear the Klebs-Löffler bacillus from the nose and throat of suspicious cases. The use of the customary Löffler coagulated beef-blood slant has necessitated the lapse of at least twenty-four hours before the clinician could have the report from the bacteriologist, and nearly always such a delay without antitoxin in positive cases is decidedly against the patient's prompt recovery from diphtheria and places the heart and other important organs in immediate jeopardy from the effects of the circulating, soluble diphtheria toxin which acts so quickly to do its irreparable damage.

With the classic Löffler slant now in common use, a lapse of fully twenty-four hours or longer was necessary for the bacilli to multiply in sufficient numbers to be recognized by the usual staining and microscopy, because in this type of tube, there is usually quite a large area of coagulated beef-blood slant and condensed moisture to be occupied by the organisms for ready and accurate recognition in the finished stained specimen. Such a long time was necessary between the taking of the nose and throat culture by the clinician and the laboratory report by the bacteriologist because of one hindering factor—a small amount of nose or throat organisms on a small sterile swab in a large amount of culture media and a sufficient amount of time allowed for the organisms to multiply in sufficient quantities for the bacteriologic examination. Possibly a second delaying or hindering factor is often present in the stock culture tubes whether they be kept carefully in the laboratory under refrigeration or carelessly in the drug store on a counter or hot, dusty shelf, and that factor is inadequate or absence of moisture in the tube, which is such a common occurrence when the attempt is made by the manufacturer to close the culture tube with the usual paraffined cork.

Ever bearing in mind these delays in and hindrances to bacterial growth, at the same time surmising any and all difficulties that might arise from physicians attempting to wait for the bacteriologic verdict, and ever bearing in mind the dangers of the culture tube without sufficient moisture for bacterial growth, we dared to depart from the use of the time-honored or classic Löffler slant and to change the method of making and preserving these culture tubes rather radically. The amount of uncoagulated beef-blood serum in the classic Löffler slant was reduced from 5 to 8 c.c. in a medium-sized test tube to 1 c.c. or less in a

\*From the City Department of Health, Greenville.  
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very small container. Instead of using the customary small and rather tightly wrapped cotton-tipped applicator, we began to use a much larger and less tightly wrapped combination. Following these somewhat drastic changes we have a large amount of nose and throat secretion on a larger and more loosely wrapped cotton tipped applicator with which to inoculate a relatively much smaller amount of culture media sealed with fresh, tightly fitting rubber corks, the evaporation from which is almost nihil. Instead of inoculating the slant with the applicator and discarding the applicator, it was retained by breaking off the stick near the cotton tip and allowing it to remain in the water of condensation at the bottom of the slant when the culture tube is held in the upright position. Such inclusion of the original applicator insures the inoculation of the slant with all of the bacteria on the tip of the applicator. Using this relatively small volume of culture media and large amount of nose and throat secretions instead of the large volume of culture media and small amount of nose and throat secretions as in the old classic Löffler culture tube, the bacteriologic laboratory is enabled to report to the clinician the microscopic examination of the stained slide in a very short time, after three to four hours' incubation at body temperature, 37.6° C.

*Method of Preparation of Culture Tube.*—1. The Löffler coagulated beef-blood medium (Difco) is prepared in accordance with instructions on the label of the dehydrated product of the Digestive Ferments Co., with one exception—1 c.c. instead of 5 c.c. is placed in a very small rubber stoppered tube.

2. One-half dram homeopathic vials or even smaller containers are used, slanted to the desired angle and sterilized in accordance with directions on the label of dehydrated beef-blood medium.

3. Soft, fresh, pliable, and tightly fitting rubber stoppers are first sterilized in 70 per cent alcohol for fifteen minutes, and then placed in the autoclave with large ends of the stoppers up for sterilization with the culture media.

4. The little vials must be stoppered immediately to prevent the loss of the water of condensation in them. If only a small amount of water of condensation remains, a few drops of sterile water must be added before stoppering.

5. Store with or without refrigeration, tightly stoppered.

*Method of Taking the Culture, Incubation, and Practical Advantages of the Vial Löffler Culture Tube.*—1. Nose, throat, and other cultures (as this medium can be used for general purposes) are taken in the usual manner.

2. The end of the culture swab is broken off short and left in the bottle to conserve all bacteria from the original culture.

3. The rubber stopper is then placed in tightly to prevent loss of moisture.

4. Incubate for three to four hours at 37.6° C. After this time there are large numbers of bacteria in sufficient quantities for the diagnosis to be made from the stained slide microscopically.

#### SUMMARY

1. The disadvantages of the conventional, old type Löffler coagulated beef-blood slant have been put forth, the principal disadvantage being due to the length of time necessary between the taking of the culture by the clinician and the final report of the bacteriologist.

2. The principal advantages of the new vial type Löffler culture tube are due to a relatively large amount of nose and throat secretion inoculated into a small amount of culture media, the bacteria of which have multiplied in sufficient numbers to enable accurate bacteriologic diagnosis in from three to four hours; the necessity of conserved moisture of condensation in the tube has also been discussed.

3. The method of preparation of the culture tube has also been given in detail, and certain technical precautions have been mentioned.

4. The method of taking the culture, incubation, storage and practical advantages of the vial Löffler coagulated beef-blood slant have been described in detail.

I am very grateful to Frank P. Simpson, Alleen Carlisle, Gladys K. Mullenix, R. N., L. Bates Fuster, R. N., Florence L. Deadwyler, and a number of our local physicians for their technical assistance in the introduction and use of the vial Löffler culture tube for the more rapid bacteriologic diagnosis of certain infections.

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## OBSERVATIONS ON MAC CONKEY'S AND DESOXYCHOLATE-CITRATE AGARS FOR THE ISOLATION OF DYSENTERY BACILLI\*

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IN RECENT years culture media appear to have been more improved for the isolation of typhoid than of dysentery bacilli. Leifson<sup>3</sup> devised desoxycholate-citrate agar for the isolation of Flexner and Schmitz dysentery bacilli. Paulson<sup>4</sup> reported that plain desoxycholate and desoxycholate-citrate agars were superior to Endo's and eosin-methylene blue agars for the investigation of infectious bowel disorders. Hardy and Watt<sup>1</sup> have confirmed these observations, particularly as they applied to the isolation of Flexner bacilli. Recent modifications of Wilson and Blair's medium by Tabet<sup>5</sup> have failed to render this medium satisfactory for growth of dysentery bacilli. Jones<sup>2</sup> found brilliant green-eosin agar quite inferior to MacConkey's agar for the isolation of dysentery bacilli. Our purpose is to record some experiences with and observations on the comparative value of MacConkey's and desoxycholate-citrate agars for the isolation of dysentery bacilli from stools in cases of acute diarrheal disorders.

### PROCEDURE

Fecal specimens unpreserved or diluted in 30 per cent glycerol saline solution were seeded on plates of the two media. Although on occasion we have prepared our own media, we have generally relied upon the dehydrated products bearing Difco or BBL labels.† Usually two plates of each medium were em-

\*From the Laboratories of the Texas State Department of Health, Austin.

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†These products were obtained from the Difco Laboratories, Detroit, and the Baltimore Biological Co., Baltimore.

TABLE I  
GROWTH ON DESOXYCHOLATE-CITRATE AGAR

ORGANISM	DESCRIPTION OF GROWTH IN TWENTY-FOUR TO FORTY-EIGHT HOURS	
	LEIFSON	IRONS AND ASSOCIATES
<i>B. coli</i>	Usually considerably inhibited; colonies slowly turned red	Usually strongly inhibited; colonies slowly turned pink or red
<i>Aerogenes</i>	Usually considerably inhibited; mucoid colonies with pink centers and colorless peripheries	Usually considerably inhibited; mucoid colonies slowly turning pink or red, especially at center
<i>Enterococci</i>	No growth	No growth
<i>Alkaligenes</i>	Usually no growth, or strongly inhibited	Greatly inhibited, or very small colorless colonies
<i>Pyocyanus</i>	Large slightly grayish-green colonies	Large grayish or olive-colored colonies
<i>Proteus</i>	Smooth, nonspreading, colorless colonies; some strains slightly inhibited	Smooth, nonspreading, nearly colorless colonies
Yeast and molds	Many grew well	Slight inhibition of some; many grew luxuriantly
<i>B. morgani</i>	Complete inhibition	No growth, or very small colorless colonies
Typhoid	Fairly large, translucent, colorless colonies; some old strains inhibited	No inhibition of recently isolated strains; translucent, colorless colonies
Paratyphoid	Large, colorless, translucent or opaque colonies; data incomplete	No recently isolated strains tested; stock cultures varied
Dysentery (Flexner)	Fairly large, colorless, opaque colonies	All recently isolated strains have grown well; colorless opaque colonies
Dysentery (Alkalescens)	No growth	Marked inhibition
Dysentery (Shiga)	Stock cultures did not grow	Stock cultures were suppressed
Dysentery (Duval-Sonne)	Poor growth	Some recently isolated strains grew poorly
Dysentery (Schmitz)	Good growth	A stock culture was suppressed

ployed per specimen. A much larger inoculum was permissible on desoxycholate-citrate than on MacConkey's plates. Suspicious colonies were subcultured on Russell's and other differential media. Fermentation of rhamnose and dulcitol was helpful, that of the former for early separation of Duval-Sonne bacilli and that of the latter for early separation of alkalescens bacilli, from the Flexner group. In every instance, however, final identification rested upon specific agglutination tests.

#### EXPERIMENTAL

On MacConkey's agar most enteric organisms grow luxuriantly. Since nonlactose fermenters grow much alike, differentiation by inspection is relatively impractical. Most nonlactose fermenters grow readily in colorless or

TABLE II

COMPARATIVE RESULTS ON SELECTED STOOLS CULTURED ON MACCONKEY'S AND DESOXYCHOLATE-CITRATE AGARS

NO.	SOURCE	MAC CONKEY'S	DESOXYCHOLATE-CITRATE
285	Bloody dysentery	Numerous colon and 5 or 6 Flexner colonies	A few colonies of aerogenes and numerous colonies of Flexner bacilli
286	Bloody dysentery	Numerous colon and 3 or 4 Flexner colonies	Many small lactose fermenters and about 50 Flexner colonies
117	Sudden diarrhea	Numerous colon colonies; nonlactose-fermenting colonies not found on 3 plates	Ten or 12 Flexner colonies and a few colonies of aerogenes
851	Bloody dysentery	Numerous Flexner and colon colonies	Numerous Flexner, aerogenes, and pin-point colonies
904	Bloody dysentery	Numerous colon, many proteus and alkaligenes colonies	Numerous proteus, pin-point and unidentified opaque colorless colonies
642	Sudden diarrhea	Numerous colon, alkaligenes, and several unidentified colonies	Numerous pin-point and unidentified colorless colonies
966	Sudden diarrhea	Numerous colon and several Flexner colonies	Several large grayish colonies; many pin-point and many Flexner colonies
957	Sudden diarrhea	Numerous colon, a few proteus, and 2 or 3 Flexner colonies	Many colon, proteus, and several Flexner colonies
426	Bloody diarrhea	Numerous colon and a few Duval-Sonne colonies	Many pin-point colonies; dysentery colonies not found
287	Sudden diarrhea	Several colon, many pyocyanus, and some other unidentified colonies	Very heavy growth of pyocyanus colonies
180	Sudden diarrhea	Numerous colon colonies; nonlactose fermenting colonies not found	Several Flexner colonies and small colonies of lactose fermenters
512	Severe diarrhea	Numerous colon and a few proteus colonies; Flexner colonies not found	About 2 dozen Flexner and many unidentified colonies
820	Sudden nausea and diarrhea	Several alkalescens dysentery and many colon colonies	A few colonies of aerogenes and pin-point colonies
146	Sudden severe diarrhea	Many colon and several Flexner colonies	Many Flexner and some unidentified colonies
856	No data	Numerous colon but Flexner colonies not found	Two or 3 Flexner colonies and several colonies of aerogenes

yellowish colonies surrounded by a clear zone, while lactose fermenters form red or pink colonies surrounded by a cloudy zone.

From the results as shown in Table II, it is evident, where Flexner bacilli were recovered, that in nearly every instance growth was more abundant on desoxycholate-citrate than on MacConkey's agar. Flexner bacilli would not have been found in Nos. 117, 180, 512, and 856 if desoxycholate-citrate agar had not been employed. Specimen No. 820 is interesting since it apparently involved a rather severe case of *B. alkalescens* food poisoning.

During the past several months 59 stools have been received from 49 individuals, suffering from bacillary dysentery or diarrhea of sudden onset, and considered possibly suitable for cultural study. Dysentery bacilli were obtained from 39 specimens. One *alkalescens* and two Duval-Sonne cultures are included. *Endameba histolytica* was found in only one of these specimens.

TABLE III  
COMPARATIVE FINDINGS WITH REGARD TO FLEXNER CULTURES

NO. OF EXAMINATIONS	CULTURES FROM MAC CONKEY'S AGAR	CULTURES FROM DESOXY- CHOLATE-CITRATE AGAR
56	20 or 36%	35 or 62%

It is interesting to note that in only one instance were Flexner bacilli found on MacConkey's when the corresponding desoxycholate-citrate plates gave a negative result.

Colonies superficially resembling those of dysentery bacilli, but which obviously lacked other cultural characteristics of the group, frequently were found in large numbers on desoxycholate-citrate plates. This disadvantage can be largely surmounted, we believe, by insisting on avoidance of gross contamination in collection of specimens.

Organisms having cultural and fermentative reactions characteristic of Flexner dysentery bacilli, but lacking in agglutinability, were rarely found. Antisera prepared against two locally obtained and carefully identified Flexner cultures generally sufficed for identification of new cultures. Frequently these two antisera gave only a slight cross agglutination.

#### DISCUSSION

During the course of this investigation Flexner bacilli were most frequently encountered and for their isolation desoxycholate-citrate agar definitely was superior to MacConkey's medium. This superiority seemed to depend upon inhibition of colon bacilli by desoxycholate-citrate medium, consequently permitting a relatively heavier seeding than was possible on MacConkey's medium. We have given considerable attention to descriptions of colonies, particularly those which are most often to be considered in inspecting desoxycholate-citrate plates, since unfamiliarity with types of growth and colonial characteristics is likely to be an important source of error and discouragement.

Since neither of two recently isolated Duval-Sonne cultures grew appreciably on desoxycholate-citrate medium, it will require further study to determine whether this medium is most suitable for cultivation of Duval-Sonne bacilli. The suppression of *alkalescens* bacilli on desoxycholate-citrate medium may not be advantageous if one departs from the usual viewpoint and considers these organisms of frequent significance.

All strains of dysentery bacilli, including stock cultures, which we have tested, have grown well on MacConkey's medium. We have found that it was wise to do confirmatory tests with colonies obtained from MacConkey's plates, or to use the utmost care in picking colonies from desoxycholate-citrate plates from which mixed cultures are all too easily obtained.

We have not encountered non-mannite-fermenting species, such as Shiga and Schmitz bacilli. None of our unidentified cultures were Newcastle bacilli.

### CONCLUSIONS

The clinical consideration of acute diarrheal disorder should include bacteriologic investigation. In this study Flexner dysentery bacilli were cultured from a high percentage of stools obtained from patients suffering from acute diarrheal disorders. Desoxycholate-citrate medium was superior to MacConkey's agar for the isolation of Flexner dysentery bacilli.

Desoxycholate-citrate agar should be used in conjunction with a less restrictive medium in the quest for dysentery bacilli. Where typhoid and paratyphoid bacilli are to be sought, other and more suitable media for this purpose should be used.

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## BLOOD TYPING AND CROSS MATCHING WITH PLASMA AND OXALATED ERYTHROCYTES\*

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DURING the past year I have typed and cross-matched 750 blood specimens with oxalated erythrocytes and plasma along with the routine method employing serum and cells. The blood was obtained by venipuncture and oxalated. Two or three drops were pipetted into a tube containing approximately 1 c.c. of 3.5 per cent sodium citrate. The tube containing the oxalated blood was centrifuged for four minutes. The plasma was in one tube, and oxalated-erythrocytes were suspended in 3.5 per cent sodium citrate in another tube. The procedure from then on was carried out as when using serum and cells.

*Advantages.*—1. With 750 blood samples I found a more rapid reaction present than when serum and citrated blood were used.

2. In doing routine work (such as complete blood counts, blood sugars, etc.) blood typing with oxalated blood and serum is very convenient.

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\*From the Ball Memorial Hospital, Muncie.  
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3. In typing I have been using known types 2 and 3 plasma. I find that the plasmas have higher titers than the known types 2 and 3 sera.

#### SUMMARY

1. Seven hundred fifty specimens of blood were grouped and cross-matched using plasmas and oxalated erythrocytes suspended in 3.5 per cent sodium citrate.

2. These blood groupings and cross-matchings were checked with the usual routine method using blood serum and cells suspended in 3.5 per cent sodium citrate.

3. Identical results were obtained with each method, with a more rapid reaction using the plasma and oxalated erythrocytes.

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### A RAPID TECHNIQUE FOR SYPHILIS TESTING WITH FINGER BLOOD\*

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THE simple and comparatively rapid modification of the Kahn test for syphilis, which was developed by Laughlen,<sup>1</sup> is increasingly becoming recognized as a dependable technique by clinical laboratories. Robinson and Stroud<sup>2</sup> confirmed its accuracy and there can be no doubt of its relative ease and speed as compared with other microtests, such as that of Kline,<sup>3</sup> and Eagle and Brand.<sup>4</sup> Detailed techniques for easy and rapid testing with finger and ear blood have not been described except in a few instances.<sup>4, 5</sup> These have not been as simple or as fast as the method described here, which was adapted from that previously developed<sup>6</sup> for use with quantitative drop analysis of finger blood. Since the Laughlen test seemed ideally adapted to use with small blood samples, it was used as the basic testing method. The complete method here described yields final results within a maximum of twenty minutes from the time of receiving a patient, and this time may often be appreciably shortened without sacrificing any step of the test. The advantage of this rapidity in case of emergency blood transfusions, as well as the general desirability of using capillary blood, e.g., with infants, is obvious. The simplicity of the technique makes accurate results available even to the relatively inexperienced operator.

#### EXPERIMENTAL

Two to 4 small drops of blood (approximately 0.1 c.c. or less), obtained by puncture of the finger or ear, were collected in a microcentrifuge cone of standard shape, 25 mm. in length and 6 mm. in external diameter, the total capacity

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of which was not more than 0.2 ml. The cone was dropped into the cup of an air-driven microcentrifuge previously described,<sup>6</sup> capable of running at about 25,000 r.p.m.\* The tube did not require balancing, but was counterweighted by a similar tube in the opposite cup. The machine was run for three to four minutes, giving in that time ample amounts of clear serum.

The serum could be handled exactly as in the Laughlen procedure in which 1 loopful of serum is added to 2 drops of antigen. In this study a slight modification was introduced by the use of capillary pipettes of approximately known volumes for all measurements. That used for measuring antigen delivered about twice the volume of that used for serum, i.e., about 0.04 ml. of antigen and 0.02 ml. of serum. The antigen was first measured roughly into the depressions of a spherically ground slide, or better, two of the depressions of a porcelain spot plate. The serum was added to one of the drops of antigen, and a known negative control serum added to the other in all cases. The slide or spot plate was rotated in the hand to mix the serum and antigen thoroughly, and the time between additions of serum and appearance of visible clumping was noted. Usually visual observation was sufficient. In doubtful cases examination with a strong lens or the low power of the microscope was helpful. No actual disagreements were ever found between the two methods of observation. If no coagulation appeared in ten minutes, the test was negative. Strong positive reactions appeared in some cases within one minute. Tests appearing within five minutes were considered + + + +, or very strong.

One hundred and twenty-five positive bloods obtained from the California State Public Health laboratory and the Clinic of the University of California Hospital were tested along with an equal number of known negative controls from various sources. In addition to known negative controls a considerable number of test blood samples found negative by the Kahn test were run along with their corresponding controls. The correlation with the results of the Kahn test run in the laboratories mentioned was exact in all but three cases, in all of which faint positives were obtained by this technique and negatives given by the Kahn test. This compares almost exactly with the results reported by Laughlen<sup>1</sup> and by Robinson and Stroud,<sup>2</sup> though the number of cases was too small to expect an exact statistical correlation.

Some results were obtained from saline extracts of dried blood and these were also exact as checked with hospital data.

The evidence here reported indicates that a dependable and simple test for syphilis may be easily run in a total of twenty minutes or less, with finger or ear blood, using the Laughlen antigen and a small high speed centrifuge.

\*Obtainable from Microchemical Specialties Co., 2112 Berkeley Way, Berkeley, Calif.

Acknowledgments are due the Lederle A dependable Laughlen antigen; to the University Public Health Laboratory for syphilitic blood of the Mount Zion Hospital of San Francisco,

ply of absolutely ie California State Larson, formerly development.



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## THE DIAGNOSIS OF TUBERCULOSIS BY CULTURE AND GUINEA PIG INOCULATION\*

CERTAIN METHODS EMPLOYED AT THE LOS ANGELES COUNTY GENERAL HOSPITAL

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INOCULATION of guinea pigs for the diagnosis of tuberculosis has long been regarded as a method of great value and sensitivity. Cultural diagnostic methods have, however, only come into prominence within recent years. This report involves mainly the comparative results of diagnostic cultures and guinea pig inoculations for the identification of *Mycobacterium tuberculosis* that were run in parallel at the Los Angeles County General Hospital over a period of seven years (October 21, 1931, to October 21, 1938). Many types of specimens were submitted for diagnosis. Among these were the following:

(a) Sputum. These specimens were generally supposed to come from patients whose sputum had been negative for acid-fast rods on six smears.

(b) Urine. This was sometimes bladder specimens and sometimes catheterized separate samples from the right and left ureters.

(c) Feces, pus from draining abscesses or sinuses, biopsy or autopsy tissue specimens. All the above types of material were generally regarded as contaminated with organisms other than *M. tuberculosis* and were, therefore, treated to get rid of these contaminants before culturing and often also before animal inoculation.

(d) Specimens potentially sterile save for possible presence of *M. tuberculosis*. In this class were included spinal fluid, pleural fluid, peritoneal fluid, and fluid or pus aspirated from joints or other closed cavities. These specimens were generally not treated for contaminants but were inoculated as received into cultures and guinea pigs after a preliminary Gram stain had failed to disclose contaminating organisms.

\*From the Laboratories of the Los Angeles County General Hospital and the Department of Bacteriology, School of Medicine, University of Southern California, Los Angeles.  
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Herrold's medium was mainly employed for the cultures. This egg yolk agar medium was made up at first according to Herrold's original description.<sup>1</sup> Later the yolks of two eggs, instead of one egg yolk, were added to 150 c.c. of agar. The technique of making cultures, although modified slightly from time to time, was in effect as follows:

Material to be cultured was mixed with from one to two volumes of 5 per cent oxalic acid. This mixture was shaken vigorously by hand and incubated at 37° C. for a period ranging from forty-five minutes to two hours during which interval it was shaken several times. After incubation the mixture was neutralized with sterile 10 per cent sodium hydroxide to which bromthymol blue had been added as an indicator. The alkali was added until the indicator just remained slightly blue (approximately pH 7.0). When the volume of the specimens permitted, this acid treatment and neutralization were performed in sterile 50 c.c. centrifuge tubes. With larger specimens this preliminary treatment was performed in sterile flasks and then poured into centrifuge tubes. After neutralization the material was centrifuged at approximately 2200 r.p.m. for a minimum of thirty minutes. The supernatant liquid was discarded, and the sediment was used for making cultures and guinea pig inoculations. The cultures were run in triplicate and approximately 0.2 c.c. of the sediment was inoculated into each tube. The culture tubes were then placed on a rack in the incubator so that the slanted surfaces of the culture medium were level and the inoculated material maintained its distribution over the surface of each slant. At this time of primary incubation the culture tubes were plugged with cotton or, in later instances, with sterile unparaffined corks. After about four days, however, when the inoculum had dried sufficiently to remain fixed on the slants, lightly paraffined sterile corks were substituted for the cotton plugs (or unparaffined corks) of the culture tubes in order to minimize further drying, and the three tubes of each culture were fastened together with a rubber band and stored upright in the incubator. The cultures were inspected grossly twice a week, and characteristic colonies were stained by the Ziehl-Neelsen method. Treatment with acid and subsequent neutralization were often omitted in the case of uncontaminated material.

Guinea pigs were inoculated either intraperitoneally or subcutaneously in the groin, and the volume of the inoculum generally approximated 1 c.c. The latter method of injection appears preferable as with it one may often be able to demonstrate acid-fast rods in pus from a local lesion long before the general health of the pig begins to be affected. A small proportion of the pigs was tested with tuberculin before inoculation. The ideal procedure would be to make this a routine test. It was usually possible to keep the animals in individual cages, but at times the pigs had to be placed two in a cage. The pigs were generally examined for local lesions or swollen inguinal lymph glands at weekly intervals beginning at a period four weeks after inoculation. When pus could be expressed from a local lesion, this was smeared and examined for acid-fast rods. In all events the pigs were killed and autopsied at the end of eight or nine weeks after inoculation. A pig was declared positive for the presence of *M. tuberculosis* only when typical gross lesions were presented and when smears or stained sections from these lesions showed the presence of typical

acid-fast rods. Stained sections were made only when smears proved to be negative. Very occasionally it was impossible to demonstrate acid-fast rods either by direct smear or by section. In this case, if the lesions showed microscopic tissue changes suggestive of tuberculosis, a report of the probable presence of *M. tuberculosis* was turned in.

In the seven-year period covered by this report, 2,439 specimens were tested for the presence of *M. tuberculosis* by cultures and guinea pig inoculations run in parallel. With few exceptions, only one guinea pig was used on a specimen, whereas 3 culture tubes were inoculated. Out of these 2,439 tests, 345 were positive by animal inoculation, culture, or both methods. Table I shows the distribution of these positive results.

TABLE I  
DISTRIBUTION OF 345 POSITIVE TESTS FOR THE PRESENCE OF *M. TUBERCULOSIS*

GUINEA PIGS POSITIVE	CULTURES POSITIVE	BOTH CULTURES AND GUINEA PIGS POSITIVE	GUINEA PIGS POSITIVE CULTURES NEGATIVE	GUINEA PIGS NEGATIVE CULTURES POSITIVE
301 87%	242 70%	198 57%	103 30%	44 13%

The results of these tests add evidence to the following suppositions which are upheld by many workers.

(a) Neither guinea pig inoculations nor cultural methods are infallible tests for the presence of *M. tuberculosis*. Both methods should be run in parallel whenever possible.

(b) Guinea pig inoculation appears to be a more sensitive method than culturing for the detection of *M. tuberculosis*.

In an attempt to determine upon a suitable culture medium, a series of parallel cultures of different specimens was run using both Herrold's egg agar medium and Corper's gentian violet potato medium.<sup>2</sup> By the time 24 specimens had grown up on Herrold's medium only 9 were positive on Corper's potato medium. Herrold's medium was then likewise compared with Corper's inspissated glycerol egg yolk medium.<sup>3</sup> Herrold's medium again came out ahead with 16 positive results against 8 for Corper's. After this comparison, Herrold's medium was used almost exclusively.

Herrold's medium can be very easily prepared. It has, however, certain theoretical disadvantages.

(a) It can be easily contaminated and contamination can sometimes occur by organisms originating within the egg inasmuch as this medium is not sterilized after the egg yolk is added. This objection has not proved serious in our experience, and very few batches of culture medium appear to have become contaminated in this manner.

(b) Egg yolk media dry out fairly easily. This tendency has been minimized by the use of paraffined corks as already described.

(c) Certain eggs may be infected with avian tubercle bacilli. If such eggs are used for making Herrold's medium, and the medium is then incubated, colonies of acid-fast rods may appear on it which are morphologically indis-

tinguishable from human or bovine strains of *M. tuberculosis*. In order to check on this possibility 7 of the positive cultures, in instances where the corresponding guinea pigs remained negative, were reinoculated into fresh guinea pigs, and all of these animals developed typical tuberculosis. We are aware, however, that various acid-fast saprophytes may at times grow up on cultures and confuse the observer. This is an occurrence which can be more easily controlled if cultures and pigs are run in parallel.

Positive results generally appear faster by culture than following guinea pig inoculation. Table II shows the distribution of times required for cultures and guinea pigs to become positive.

TABLE II  
DISTRIBUTION OF TIME INTERVALS REQUIRED FOR CULTURES AND GUINEA PIGS TO SHOW THE PRESENCE OF *M. TUBERCULOSIS*

CULTURES	TIME INTERVALS	GUINEA PIGS
5 ( 2 per cent)	Less than 2 weeks	
7 ( 3 per cent)	2 weeks	
46 (19 per cent)	3 weeks	
75 (31 per cent)	4 weeks	7 ( 2 per cent)
47 (20 per cent)	5 weeks	9 ( 3 per cent)
42 (17 per cent)	6 weeks	37 (12 per cent)
13 ( 5 per cent)	7 weeks	62 (21 per cent)
5 ( 2 per cent)	8 weeks	158 (53 per cent)
	More than 8 weeks	28 ( 9 per cent)
2 ( 1 per cent)	Unrecorded times	
242	Totals	301

The use of cultures as a routine diagnostic method for tuberculosis requires considerable experience in order to obtain the best results. This is brought out in the series here reported when the 345 tests positive by culture, guinea pig inoculation, or both methods (Table I) are divided into (a) the first 150 positives and (b) the last 195 positives, upon which it appears that 93 out of the first 150 tests, or 62 per cent, gave positive cultural results, whereas 149 out of the last 195, or 76 per cent, showed growth of acid-fast rods on culture. The earlier records of our results fail to show the number of cultures that were reported as negative for *M. tuberculosis* because they had become contaminated by extraneous organisms introduced with the inoculum. In the last group of 1694 cases, however, 38 cultures (considering the 3 tubes as one culture) or 2 per cent of this series had to be discarded due to such contamination.

Guinea pig inoculation did not prove as uniformly efficacious a method for the identification of *M. tuberculosis* as we had previously considered it to be. In the first 150 of the 345 positive tests 139, or 93 per cent, of the pigs were positive, but only 162 out of the second 195, or 83 per cent, gave positive results. This drop in the efficiency of the guinea pig method as the series progressed was disappointing. It can probably be explained in part by the fact that an epidemic was killing a good many of the hospital's supply of pigs for a time and that, therefore, a number of pigs died too soon after inoculation to show definite positive or negative results. It was obvious, however, that every so often positive cultures would correspond with a pig that appeared

quite normal when autopsied eight weeks after inoculation; and, as stated previously, certain of these cultures on reinoculation into other guinea pigs produced typical tuberculosis. It is possible that these discrepancies may be accounted for by the distribution of organisms in the original suspected material. Our laboratory had no shaking machine to thoroughly homogenize the original specimens. A thorough shaking of these specimens with the acid used to eliminate contaminants should, in our opinion, eliminate a fair percentage of the discrepancies encountered between culture and guinea pig inoculation.

Murphy and Duerschner<sup>4</sup> (1938) reported a carefully performed comparison between the efficacies of cultural and guinea pig inoculation methods for the diagnosis of tuberculosis. They used slants of Petragnani's medium and inoculated them in triplicate with a total volume of material equal to that which was injected into the corresponding guinea pigs. Their results indicated that the cultural method possessed a sensitivity slightly greater than that shown by the method of guinea pig inoculation. One per cent of their complete culture sets had to be discarded due to contamination. They noted that the cultures became positive by microscopic examination about one week before macroscopic colonies appeared.

The results herewith reported do not uphold the contention that cultural methods are more sensitive than the inoculation of guinea pigs for the detection of *M. tuberculosis*. In evaluating our results, however, it must be remembered that the guinea pigs generally received larger volumes of inoculated material than that placed into the corresponding culture sets. When our positive tests are broken down into different groups of 50 consecutive cases, the following results obtain:

CASES		POSITIVE GUINEA PIGS	POSITIVE CULTURES
First	50	47	31
Second	50	46	31
Third	50	46	31
Fourth	50	38	40
Fifth	50	41	34
Sixth	50	47	41
Last	45	36	34

If the fourth 50 cases had been studied alone one would gain the impression that culturing was the most sensitive method, although the whole series gives evidence in favor of the guinea pig technique. It is possible that the series of Murphy and Duerschner might also have pointed in this direction had it been somewhat larger. The routine weekly microscopic examination of cultures before the appearance of gross colonies, as performed by Murphy and Duerschner, would appear to be an excellent technique to follow if time permitted.

One of us (Fisk) working in the laboratory of the contagious unit of the hospital, has developed a method of culturing spinal fluids which has proved useful in growing out many infectious agents including *M. tuberculosis*. The spinal fluid from a case of suspected meningitis is centrifuged at high speed for approximately thirty minutes. The supernatant liquid is discarded, and real infusion broth containing 20 per cent filtered inactivated human serum is

poured over the sediment. The tube is then tightly corked and incubated. Among the pathogenic agents recoverable from spinal fluid by this method are meningococci, alpha and beta streptococci, staphylococci, pneumococci, *H. influenzae*, diphtheroids, *Coccidioides immitis*, and *Mycobacterium tuberculosis*. Most of these organisms appear rapidly. *M. tuberculosis*, however, takes two weeks or more to develop gross colonies. These colonies grow in the bottom of the culture and look like little flecks of wax. On smear they are composed of typical acid-fast rods. Several of these positive cultures have been inoculated into guinea pigs and have always brought about typical tuberculosis. All cultures positive for *M. tuberculosis* have corresponded to cases that were diagnosed as tuberculous meningitis either clinically or at autopsy. A large control series of spinal fluids taken from cases of various types of encephalitis, poliomyelitis, and various forms of neurosyphilis have failed to show growth of *M. tuberculosis* or other saprophytic acid-fast rods. This method for culturing many types of pathologic spinal fluids is reported here because it works very well in the case of tuberculous meningitis and because of its general applicability.

#### SUMMARY

Parallel cultures and guinea pig inoculations for the identification of *Mycobacterium tuberculosis* were run over a period of seven years. The animal inoculation method appeared to be the most sensitive. However, neither cultural nor animal inoculation methods were absolutely reliable, and it is suggested that both be performed as a check on one another whenever possible.

A simple method for culturing spinal fluid is described. This method favors the growth of many pathogens including *Mycobacterium tuberculosis*.

We gratefully acknowledge personal advice concerning the laboratory diagnosis of tuberculosis from Doctors H. J. Corper, Joseph E. Pottenger, C. Richard Smith, and Emil Bogen.

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## DEMONSTRATION OF RABIES VIRUS IN GROSSLY DECOMPOSED ANIMAL BRAINS\*

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PUTREFIED brain tissue is usually difficult material in which to demonstrate Negri bodies<sup>1</sup> and for this reason the diagnosis of a decomposed brain by microscopic examination is unreliable. It is obvious, therefore, that a suitable and rapid animal inoculation method is desirable in order to obviate these difficulties. Only few attempts, however, have been made to sterilize decomposed animal brains without affecting the virus which is known to remain virulent in putrefied brain tissue for several days or even months.<sup>2, 3</sup> Mazzei,<sup>4</sup> for example, was able to demonstrate rabies virus in Berkefeld filtrates of tissue which had undergone putrefaction for sixty-three days. Other investigators<sup>5, 6</sup> have also successfully filtered rabies virus, but due to the inconsistency of the results this method is impractical in a health department laboratory.

In order to attempt isolation or demonstration of virus in decomposed brains it became necessary to remove the bacterial contaminants from the putrid material since intracerebral injection of such material into mice invariably produced death of the animal from intercurrent infection or from brain abscess.

In the preliminary experiments several bactericidal agents, including merthiolate, glycerin, phenol, and ether, were tried in an effort to find one which would sterilize the heavily infected inoculum without appreciably affecting the virus. Ether used according to the method suggested by Kramer and his associates,<sup>7</sup> Paul and Trask,<sup>8</sup> and others, proved to be the most desirable agent because its extreme volatility permitted prompt removal from the contaminated material after its bactericidal action had taken effect.

The following experiment was carried out to determine the bactericidal effect of ether upon contaminated dog brains. The horn (cornu ammonis) was removed from a grossly contaminated dog brain and ground in a mortar without an abrasive. Nutrient broth was added to make an approximate 20 per cent suspension which was then centrifuged at slow speed for about five minutes. The supernatant liquid was then divided into 7 aliquot portions, to 6 of which was added anesthetic ether to final concentration of 3, 6, 10, 12, 15, and 20 per cent, respectively. No ether was added to the first tube which served as control. The tubes were then thoroughly shaken, placed in the icebox (4° C.) and removed at intervals of one, two, six, eighteen hours to be cultured both aerobically and anaerobically on blood agar. The results of this experiment, sum-

\*From the Laboratory Section of the St. Louis Health Division, St. Louis, Missouri.  
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marized in Table I, and representing the average findings of several experiments, indicate that ether in final concentration of 10 per cent exerts a definite bactericidal effect upon contaminated dog brains after exposure for two hours at 4° C.

Having determined the optimum concentration of ether necessary to destroy contaminants in decomposed brains, it became necessary to establish whether this amount of ether would have any effect upon the virus.

TABLE I  
BACTERICIDAL EFFECT OF ETHER UPON CONTAMINATED DOG BRAINS

	CONTROL NO ETHER		CONCENTRATION OF ETHER											
			3%		6%		10%		12%		15%		20%	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Exposure to ether at 4° C. for														
1 hour	+++	+++	+++	+++	+++	++	++	+	0	0	0	0	0	0
2 hours			++	++	++	++	0	+	0	0	0	0	0	0
6 hours			++	++	++	+	0	0	0	0	0	0	0	0
18 hours	+++	+++	++	+++	++	0	0	0	0	0	0	0	0	0

1 = 1 loopful plated on blood agar and cultured aerobically.

2 = Cultured anaerobically.

0 = No growth.

+= 1-20 colonies.

++ = 20-100 colonies.

+++ = Heavy growth.

It is quite definitely established that ether has an attenuating effect upon the rabies virus only after long exposure.<sup>9-12</sup> Remlinger and his co-workers,<sup>10</sup> for example, studied the effect of ether on different strains of street and fixed virus and found that the brain tissue from the center of the hippocampus major (cornu ammonis) remained virulent after immersion in undiluted ether for one hundred twenty hours, while one strain of fixed virus remained virulent for one hundred sixty-eight hours.

The following experiment was designed to establish the concentration of ether that would have a definite effect upon the virulence of the virus after contact for eighteen hours. The horn (cornu ammonis) was removed from a dog brain suspected of being rabid. Two small pieces of horn were removed for microscopic examination (impression smears), and two additional pieces were preserved in full strength glycerin. The remaining portion of both horns was ground in a mortar without an abrasive, and sufficient nutrient broth was added to make an approximate 20 per cent suspension. After standing at room temperature for one hour, the suspension was centrifuged at slow speed in order to remove the large clumps. The supernatant liquid was then divided into 6 aliquot portions dispensed in sterile stoppered 15 c.c. centrifuge tubes. To 5 of these tubes was added anesthetic ether to final concentration of 7, 10, 15, 20, 30 per cent, respectively. The first tube received no ether and served as control. After thorough shaking, the tubes were placed in the icebox (4° C.) for eighteen hours, at which time they were centrifuged, and the supernatant liquid below the ether layer from each tube was injected intracerebrally into



each of 4 mice. In order to insure complete removal of the ether before the intracerebral injection was made, the tubes were placed in a desiccator for about five minutes.

TABLE II

EFFECT ON VIRUS AFTER EXPOSURE TO VARIOUS CONCENTRATIONS OF ETHER FOR 18 HOURS AT 4° C.

EXPOSURE TO DIFFERENT CONCENTRATIONS OF ETHER FOR 18 HR. AT 4° C.	DAY OF DEATH AFTER INTRACEREBRAL AND INTRAMUSCULAR INJECTION OF VIRUS	
	STREET VIRUS NO. 2715	STREET VIRUS NO. 2717
7 per cent	8+, 10+, 13,** 16+	4,** 9+, 11+, 16+
10 per cent	9+, 11+, 11+, 14**	8+, 11+, 13+, 13+
15 per cent	9+, 10+, 12+, 21+	9+, 9**, 12+, 14+
20 per cent	12+, 13+, 18+, 19+	1,* 15+, 15+, 18+
30 per cent	1,* 16+, 19+, 21-	17+, 21,** 26+, S
Control—No ether	8+, 10+, 10+, 15+	2,* 12+, 14+, 14+

\*Animal died from trauma or of unknown complication other than rabies.

\*\*Animal found dead and partially devoured. Examination could not be made.

+Negri bodies found on microscopic examination.

-No Negri bodies found on microscopic examination.

S = Animal survived (six weeks).

The results of this experiment, summarized in Table II, indicate that exposure to 10 per cent ether for eighteen hours at 4° C. had no effect upon the virulence of the two strains of street virus tested. Exposure to 30 per cent ether only slightly altered the virulence of these strains.

Having established that 10 per cent ether exerts a definite bactericidal effect upon contaminated dog brains after exposure for two hours at 4° C., and having determined that this concentration of ether does not effect the rabies virus under these conditions, a series of experiments was carried out using dog brains proving positive on microscopic examination. These brains were allowed to stand at room temperature until grossly contaminated. The experiments were conducted with the view of establishing a simple and practical method for treating contaminated brains before intracerebral injection into animals. The procedure used was as follows: The central portion of the hippocampus major (cornu ammonis) was dissected out, and a small piece was removed for microscopic examination and for glycerination. The remaining portion of the horn was emulsified in 10 c.c. of nutrient broth and allowed to stand for one hour at room temperature. The supernatant liquid (after larger clumps settled) was pipetted into a stoppered 15 c.c. centrifuge tube, and 10 per cent ether (about 1.0 c.c.) was added. The tube was then shaken thoroughly and allowed to stand in the refrigerator for two hours. After centrifugation at slow speed the supernatant liquid below the ether layer was removed for animal inoculation.

Nine grossly contaminated positive dog brains have been examined by the method described, and in each case a confirmatory diagnosis of rabies could be made by the mouse inoculation method.<sup>13</sup> The results summarized in Table III show that a positive report could be sent to the rabies clinic in an average of ten days after the animal was brought to the laboratory for examination. The method described has already been found practical in one specimen submitted for examination. A dog was brought to the laboratory on Aug. 15, 1938, and no satisfactory microscopic examination of the brain (No. 2741) could be made

because of gross decomposition. A positive report was sent to the rabies clinic on Aug. 26, 1938, eleven days after the ether-treated brain was injected intracerebrally in mice.

TABLE III

GROSSLY CONTAMINATED DOG BRAINS SHOWING NEGRI BODIES, EXPOSED TO 10 PER CENT ETHER FOR TWO HOURS AT 4° C.

DOG BRAIN NO.	DAY OF DEATH AFTER INTRACEREBRAL AND INTRAMUSCULAR INJECTION OF ETHER-TREATED DOG BRAINS			
2741†	11+,	13+,	13,**	15+
2743	9+,	10+,	12+,	12+
2747	1,*	4-,	12+,	S
2752	9+,	9+,	9+,	12+
2757	5-,	9+,	11+,	14+
2774	9+,	10+,	10+,	S
2793	10+,	10+,	12+,	12+
2796	9+,	10+,	10,**	11+
2800	11+,	13+,	13+,	14+
2803	1,*	10+,	11+,	12+

\*Animal died from trauma or of unknown complication other than rabies.

\*\*Animal found dead and partially devoured. Examination could not be made.

†Negri bodies found on microscopic examination.

-No Negri bodies found on microscopic examination.

‡No satisfactory examination could be made of this brain because of gross decomposition.

S = Animal survived.

## SUMMARY

Experiments are presented describing a simple procedure for demonstrating rabies virus in grossly decomposed animal brains. Of several bactericidal agents, including merthiolate, glycerin, phenol, and ether, the last mentioned proved to be the most desirable. It was found that ether in final concentration of 10 per cent exerts a definite bactericidal effect upon contaminated dog brains after exposure for two hours at 4° C. Exposure to this concentration of ether for eighteen hours at 4° C. had no effect upon the virulence of two strains of street virus tested. The experiments were conducted with the view of establishing a simple and practical method for treating contaminated brains before intracerebral injection into animals.

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## CLINICAL DEMONSTRATION OF IRON IN THE SKIN IN HEMOCHROMATOSIS\*

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THE diagnosis of hemochromatosis is verified by the finding of iron deposited diffusely in the skin. The visual evidence of its presence, that is, the pigmentation, may vary considerably in intensity and in its shading from light yellow brown, deep bronze, or bluish brown, to slate color. The two conditions with pigmentation most likely to be confused with that of hemochromatosis are Addison's disease and argyria. While clinical study may serve in differentiation, yet a positive ruling on the presence of dermal iron is the simplest and most direct finding.

The histochemical demonstration of iron has been done by the excision of a piece of skin, fixing it, sectioning, and exposing the sections to the action of chemicals which develop the iron colorimetrically. While this is not a formidable procedure, it is something of a nuisance to both the patient and the doctor, and consequently is used very seldom. The time required for a report may vary, but under common working conditions it generally ranges from two to five days.

A man with hemochromatosis was seen recently with the familiar triad of findings: pigmentation of the skin with iron demonstrated histologically, diabetes mellitus, and enlarged cirrhotic liver. Some of the disadvantages of the old procedure for demonstrating iron in the skin were brought to attention and a new method was devised.

Equal parts of sterile solutions of 0.5 per cent potassium ferrocyanide and one-hundredth normal hydrochloric acid were mixed and injected intradermally so as to form a wheal.

A slight blue color was evident almost immediately, which darkened to a deep blue within five minutes. It is possible that the color became somewhat darker over the next few hours, but the change was insignificant. A narrow red zone appeared at the periphery of the wheal in about two days. This persisted throughout the slow contraction of the blue test spot until its disappearance in about two weeks.

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The negative test showed a white wheal which later developed the peripheral red zone and disappeared in about the same time as a positive test.

There is a sharp stinging sensation with the injection, but it ceases within a few seconds. There is no pain or itching afterward, as might be developed with acute inflammation. The skin remains intact without peeling or necrosis.

Hemorrhage into the tissues from bruises or other cause might result in iron deposit, but these causes are local and usually can be ruled out easily.

In post-mortem examination in the case here cited the iron test was positive in the skin of any chosen area of the body or extremities, even in parts where no altered pigmentation was visible. It is more difficult to inject the solution intradermally after death, although such localization is necessary for development of the color.

#### SUMMARY

A test for iron in the skin is described, making a simple application of the familiar potassium ferrocyanide chemical reaction for iron.

It is without any evident injurious local or general effects.

The test can be performed and read within a few minutes in the doctor's office.

Since diffuse iron deposit occurs only in hemochromatosis, this test would appear to be specific for that disease.

In hemochromatosis the test is also positive after death.

Grateful acknowledgment is made here to the Davella Mills Cancer Foundation Fund of Wesley Memorial Hospital of Chicago, which provided hospital care for the patient studied.

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### A RING TEST FOR URINE BROMIDES\*

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**I**N MENTAL patients the question of bromism arises frequently.<sup>1</sup> Wuth<sup>2</sup> in 1927 described a test to detect the presence of bromides in the urine as follows:

To 25 c.c. of urine add 1 gm. of animal charcoal. Mix well. Allow to stand for a few minutes and filter. To exactly 5 c.c. of filtrate add 1 c.c. of 20 per cent trichloroacetic acid and 1 c.c. of 0.5 per cent gold chloride. A brown shade indicates the presence of bromides. Iodides will be indicated by a precipitate. For comparison make the following solution: 0.5 c.c. of 0.1 per cent sodium bromide, 4.5 c.c. of water, 1 c.c. of 20 per cent trichloroacetic acid and 1 c.c. of 0.5 per cent gold chloride.

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It seems to us that a simpler procedure than that recommended by Wuth is desirable for routine clinical usage. The following ring test has proved satisfactory in our hands:

Roughly 1 c.c. of 20 per cent trichloroacetic acid is mixed with approximately 5 c.c. of urine, and the mixture is overlaid with 0.5 per cent gold chloride.

If bromide is present in concentrations over 50 mg. in 100 c.c. of urine, a yellowish to a reddish orange ring develops almost immediately at the interface. Occasionally the ring will have a brownish tinge, but that is not the predominant color. The characteristic color reaction is not obscured even in highly pigmented urines. The test is difficult to read in artificial light unless a daylight bulb is used. To become familiar with the color of the ring, one need only perform the test on urines containing various concentrations of bromides. At 50 mg. per cent a positive test first appears as a canary yellow ring; at 80 mg. per cent, the color obtained is orange; at 160 mg. per cent, the ring is reddish orange. With higher bromide concentrations the reddish orange ring becomes more intense. Not infrequently a grayish precipitate forms at the interface of the gold chloride and urine, and occasionally a purple color appears below the interface. These reactions should not be interpreted as positive tests in the absence of the characteristically colored ring. Bile and iodides, if present in the urine, interfere with the test. Urines containing a high concentration of bile and bromides give a negative test. Iodides in a urine containing bromides mask the color of the ring by a yellowish gray precipitate which is immediately formed. The iodide reaction is readily distinguishable from the bromide reaction, but it will mask bromides if present.

In order to determine the clinical limitations of the procedure, the ring test was done on urines of patients receiving therapeutic doses of sodium bromide. Five patients received 0.6 gm. of sodium bromide three times a day. Urine tests became positive on the fourth day of medication in 2 cases; on the fifth day in 2 cases, and on the seventh day in one case. Twenty patients received 1 gm. sodium bromide three times a day. The urine test was positive after one day of medication in 2 cases; two days in 6 cases; three days in 6 cases; four days in 4 cases; and five days in 2 cases. In no instance did the urine test remain negative following the usual therapeutic medication.

The test was done routinely on urines from patients on the medical wards of the Psychiatric Division of Bellevue Hospital. Nine hundred and sixty-six urine specimens were tested; 164 gave a positive ring test for bromides. In every positive test the patient had either been given bromides in the hospital, gave a definite history of bromide ingestion, or had over 75 mg. per cent bromides in the blood serum. Using those features as criteria, no false positives were discovered in the 966 urine specimens.

In 520 consecutive cases admitted chiefly to the medical wards of the Psychiatric Division of Bellevue Hospital, the ring test was positive for urinary bromides in 23 patients. In 13 cases bromide ingestion was not suspected before the urine bromide test was done. Simultaneous blood bromide determinations<sup>3</sup> and urinary tests were done on 97 patients. In every instance where the urine test was positive the serum bromide level was above 75 mg. per cent. Seven showed serum bromide levels above 75 mg. per cent. In only one case was

the ring test in the urine negative when the serum bromide level was above 75 mg. per cent. In this case the serum bromide on admission was 125 mg. per cent, at which time the ring test was positive. After 10 days the urinary test became negative, although the serum bromide level persisted at 100 mg. per cent. This patient was a diabetic who had bouts of vomiting and took salts and fluids poorly.

The ring test may also be done on spinal fluid and blood serum. One need only overlay spinal fluid with 0.5 per cent gold chloride solution and look for the characteristically colored ring at the interface. For a qualitative blood bromide test the serum proteins are precipitated by 20 per cent trichloroacetic acid and spun down by centrifuge. The supernatant fluid is overlaid with 0.5 per cent gold chloride. For example, in one case, the serum bromide was 200 mg. per cent; the ring tests on serum and spinal fluids were positive.

#### COMMENT

The test here described has been useful to us as a routine procedure to detect the presence of bromides in urine. When the urine test is positive, a quantitative blood bromide determination is done. In our experience a negative urine test is strong evidence against bromism. In cases of suspected bromide intoxication it offers a quick method of confirming the diagnosis. The test can easily be performed as part of the routine urine examination. The necessary solutions are stable and easily prepared.

#### CONCLUSION

A ring test for urinary bromides is described. It is suggested that the test can be used as a routine procedure on all patients entering mental hospitals, or whenever indicated in general hospitals or office practice.

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## PREPARATIONS FROM THE SPINAL CORD IN THE LABORATORY DIAGNOSIS OF RABIES\*

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IN THE laboratory diagnosis of rabies, preparations are usually made from the hippocampus major, the gray matter of the cerebellum or the cortex in the region of the fissure of Rolando, because Negri bodies usually occur most abundantly in these areas. Park, Williams, and Krumwiede<sup>1</sup> have stated that they "have always found bodies in the spinal cord in abundance, but here they are especially prone to be localized in discrete groups of cells."

To our knowledge preparations from the cord are not usually employed for the demonstration of Negri bodies. In most of the specimens received at the Arizona State Laboratory for the laboratory diagnosis of rabies, the head has already been removed, and the cord is readily accessible. It occurred to us that the use of the cord for this purpose would result in a considerable saving of time, and would be particularly valuable in cases where the animal had been shot through the head.

We have adopted the following procedure when the head has been removed: Preparations are made from the cord and are stained in the usual manner. If Negri bodies are demonstrated, no further examination is made. If Negri bodies are not found in the cord, then preparations are made from Ammon's horn. So far, the results of examinations of preparations from the cord and from Ammon's horn have agreed perfectly. We feel, however, that in the event that Negri bodies are not demonstrated in the cord, preparation should be made from the brain.

The use of the cord for this purpose results in a saving of time, particularly in the case of larger animals, where the removal of the brain is not always easy.

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## A METHOD OF COUNTING BLOOD PLATELETS

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THE numerous methods that have been proposed for counting blood platelets, and the fact that the normal count differs with the method used, seem to indicate that an entirely satisfactory method has not been devised.

Our experience indicates that the normal number of platelets varies not only with the method used, but also with the technician making the count. Where it is necessary to make a series of platelet counts upon a patient, we insist that all counts be made by the same technician.

In few, if any, laboratories is a platelet count among the procedures carried out as a routine in doing a complete count. Yet, we feel that the platelet count is of sufficient importance to warrant its inclusion in routine "complete blood counts," and that if this were done, our knowledge concerning the significance of variation in the number of platelets would be materially increased. Doubtless, if a simple, accurate method of counting platelets were available, it would be used as a routine.

We have found the method of making plasma platelet counts as devised by Nygaard the most satisfactory method in use at the present time. However, since it requires venipuncture, and since the syringe is filled with a more or less toxic solution, it is seldom used as a routine procedure. In addition, the time required for the sedimentation of the red blood cells is a disadvantage.

We have found that the majority of difficulties in making platelet counts are overcome by the use of the following method:

1. Before making puncture in finger or ear, draw a 1.1 per cent solution of sodium oxalate into white blood cell pipette to the mark 1 and expel it by blowing. This moistens the pipette and prevents platelets from adhering to the glass.

2. Quickly draw blood to the mark 0.5.

3. Quickly draw diluting fluid (1.1 per cent solution of sodium oxalate) to the mark 11, and mix by shaking vigorously.

4. Place a heavy rubber band around pipette so as to close ends.

- 5a. Place pipette in centrifuge and centrifugalize for the shortest possible time necessary to drive red blood cells into stem of pipette. (We have found that one-half minute at a speed of approximately 1,600 revolutions will accomplish this without changing the platelet count) or

- 5b. Let pipette stand upright for two hours to permit the red blood cells to settle into the stem of the pipette. (There is some variation in time depending on the caliber of the lumen in the stem of pipette.)

6. Gently expel the red blood cells from the stem of the pipette by blowing.



7. Place a drop of clear supernatant solution in counting chamber (a few red blood cells will do no harm).

8. After ten minutes count all the platelets in 80 small squares (4 groups of 20 small squares). Because of the slowness with which platelets settle, it is very important to focus carefully over each square counted so as to count platelets at different levels in the chamber.

9. Add three zeros to the total count for the number of platelets per cubic millimeter of blood. (We have considered 300,000 as the normal number per cubic millimeter, using this method.)

In step 1, we at first filled the pipette with the diluting fluid to mark 1 and then drew the blood into the pipette to the mark 0.5, as directed in some of the older methods. However, we found that the blood and the solution tended to blend, making it difficult to draw in the correct amount of blood. When the pipette was simply moistened, the blood could be accurately drawn to the mark 0.5, and the variation between counts on the same patient became less, indicating greater accuracy.

Using this method, we feel that it is possible to count platelets with a percentage of error not much greater than in counting red blood cells. The amount of work is not appreciably greater than that involved in doing a red blood cell count.

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## ZONE REACTIONS IN THE KLINE TEST\*

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IT IS frequently observed in serologic reactions that lower dilutions of serum may give negative results, while higher dilutions of the same serum may give strongly positive reactions. Zone reactions are frequently encountered in the Wassermann test (an excellent discussion is given by Eagle<sup>1</sup>), but to our knowledge have not been observed frequently in flocculation tests for syphilis, especially in those tests where a single fixed antigen-serum ratio, is employed.

At the Arizona State Laboratory the more common serologic tests for syphilis have given excellent agreement.<sup>2</sup> Occasionally, we have noted a serum which might give a doubtful or weakly positive Kline reaction and strongly positive Kahn, Hinton, or Wassermann reactions. At first these were considered as technical errors, but when several of these Kline-doubtful-Kahn-positive sera were examined in two different laboratories, by three different persons who employed different lots of LaMotte Kline antigen, we concluded that these results were not necessarily technical errors.

It appeared to us that these discrepant results might be due to a zone effect which was not appearing in the Kahn test because, in the latter, three tubes of varying antigen-serum ratio are employed. In order to determine whether these results might be due to a zone effect, these sera were diluted with physiologic saline, and the Kline test was performed upon the diluted sera. At the

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same time a large number of negative sera were diluted and examined in the same manner as the positive sera. In every case these negative sera gave uniformly negative Kline reactions.

Table I gives some typical results of the examination of these sera.

TABLE I  
KLINE REACTIONS ON DILUTED SERUM

SERUM NO.	KAHN (UNDILUTED SERUM)	KLINE (UNDILUTED SERUM)	KLINE					
			1:1	1:2	1:4	1:8	1:16	1:32
110	3 plus	1 plus	2 plus	4 plus	4 plus	2 plus	1 plus	Doubtful
630	2 plus	1 plus	1 plus	3 plus	4 plus	3 plus	2 plus	Doubtful
1508	4 plus	1 plus	2 plus	3 plus	1 plus	Doubtful	Negative	Negative
1576	4 plus	Doubtful	3 plus	1 plus	Doubtful	Negative	Negative	Negative
1681	4 plus	1 plus	3 plus	1 plus	Doubtful	Negative	Negative	Negative
1828	4 plus	Doubtful	2 plus	2 plus	3 plus	Doubtful	Negative	Negative
1855	4 plus	1 plus	1 plus	2 plus	1 plus	Doubtful	Negative	Negative
9805	4 plus	2 plus	3 plus	4 plus	3 plus	1 plus	Negative	Negative
9937	4 plus	2 plus	2 plus	3 plus	3 plus	2 plus	1 plus	Doubtful

An examination of Table I reveals that when the sera were diluted, stronger reactions were observed in the higher dilutions than in the undiluted sera. In general, the Kline results on serum dilutions of 1:1 or 1:2 agreed well with the Kahn results on the undiluted sera. Negative sera, treated in the same manner, gave uniformly negative results. Several hundred other sera, which gave comparable Kline, Kahn, Hinton, and Wassermann reactions, have been examined in the same manner, but zone effects have not been observed.

Although the Hinton test is not read in terms of degrees of positivity, we have, in a few cases, noted zone effects in diluted sera. That is, a more abundant floe is formed in the diluted sera than in the undiluted.

The incidence of the sera which show zone effects is (in our experience) not great—approximately 1 per 1,000—and should not prove a great source of error, unless only one test is employed in the laboratory diagnosis of syphilis. These results emphasize the desirability of employing more than one technique, and particularly the desirability of employing either dilutions of serum or one test in which the antigen-serum ratios are varied.

#### CONCLUSION

It was found that sera which gave strongly positive Kahn and weakly positive Kline reactions showed a zone effect when the serum was diluted with saline. In general, such sera, when diluted 1:1 or 1:2, gave Kline reactions which compared to the Kahn reactions with undiluted sera.

The incidence of these sera is not great (approximately 1 per 1,000).

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2. Breazeale, E. L., Greene, R. A., and Harding, H. B.: A Comparison of the Eagle, Ide, Kahn, Kline and Laughlen Tests for Syphilis, *Southwest Med.* 22: 311, 1938.

Since this manuscript was submitted, our attention has been called to similar observations by Dr. A. S. Wiener, *J. LAB. & CLIN. MED.* 22: 1069, 1937.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

## EMBEDDING OVEN, an Inexpensive, Bouton, S. M., Jr. Arch. Path. 27: 58, 1939.

The oven consists primarily of a box constructed throughout of  $1\frac{1}{8}$  inch (2.8 cm.) lumber, thus utilizing the insulating qualities of wood. The door occupies the entire frontage, is completely recessed into the frame of the box, and consists of a wood frame holding two panes of glass, separated from each other by an intervening air space. The door is supplied with an inexpensive catch lock. The inside of the box is equipped a short distance from the bottom with four regulation screw sockets, two in each side wall, to hold four electric light bulbs (in this laboratory, two 25 watt and two 50 watt bulbs); and the outside, with individual switches, one for each bulb. The electric cord leaves the box at the rear. The top of the box is perforated by six  $\frac{1}{2}$  inch (1.3 cm.) holes (the number and diameter of these holes may, of course, vary) within a rectangular space large enough to accommodate a dehydrating jar. This space is marked off by a low wooden frame to hold the jar; the frame, open at the rear, furnishes guides for a sliding panel which can be adjusted to cover from one to all six holes. For this purpose, the latter are staggered.

The inside of the box is further equipped with rails along both sides, not too close to the bulbs, to hold two removable stout wire mesh trays. A centigrade thermometer is hung on the inside of the door, its mercury bulb insulated against the glass by a piece of very thick cardboard. Thus the temperature within the oven can be read at a glance without opening the door. For purposes of comparison and checking, another thermometer may be attached to the rear wall in such a manner that it also may be read through the closed door.

## CLOT RETRACTION, A Simple Method for Measuring, MacFarland, R. G. Brit. Lancet 1: 1199, 1939.

The apparatus required for measuring clot retraction consists only of a graduated centrifuge tube with the scale marked in 0.1 cm. divisions, a glass rod, and a cork to fit the mouth of the tube, bored to receive the rod as an easy sliding fit. The rod is about  $\frac{1}{2}$  inch longer than the tube and has a small "button" expansion formed about  $\frac{1}{2}$  inch from one end.

The retraction is measured as follows: More than 5 cm. of blood is obtained by venepuncture. The centrifuge tube is filled from the syringe up to the 5 cm. mark. The glass rod is put into the tube, so that the expansion is immersed in the blood, and the cork is fitted, the projecting end of the rod passing through the hole bored to receive it. The tube is then placed in a water bath at  $37^{\circ}$  C. and examined from time to time for coagulation of the contained blood. An hour after firm clotting it is removed from the bath. If retraction has taken place, the clot will be seen to have shrunk away from the walls of the tube and to be attached to the rod only. By sliding the rod upwards through the cork, the clot, supported on the expansion, can be raised clear of the expressed fluid, so that the volume of the latter may be measured directly on the graduated scale.

Retraction is expressed in terms of this volume, as a percentage of the original volume of blood. Thus, if 5 cm. of blood after clotting express 3 cm. of fluid, the retraction is

$$\frac{3 \times 100}{5} = 60 \text{ per cent.}$$

In measuring retraction in this way it is necessary to ensure that the graduated tubes are perfectly clean and are unscratched inside; otherwise the clot may stick to the glass and

prevent retraction. Clots that have stuck may be freed by carefully passing a fine stiff wire round the inside of the tube, keeping it in contact with the glass. The tube should then be incubated for a further period of an hour.

From a study of 50 normal donors (23 female, 27 male) the normal range by this method was 43.9 to 66.5 per cent.

In a series of 250 pathologic cases it was found that retraction might be reduced in jaundice, lobar pneumonia, myelomatosis, and other conditions, apart from those with an obvious reduction in the platelets.

In the hemorrhagic states, retraction was normal in hemophilia, telangiectasis, and athrombopenic purpura hemorrhagica. Only in thrombopenic purpura was it reduced.

**SULFANILAMIDE.** A Study of Its Mode of Action on Hemolytic Streptococci, Keefer, C. S., and Rantz, L. A. Arch. Int. Med. 63: 957, 1939.

From a study of the mode of action of sulfanilamide *in vitro* and *in vivo*, the following facts emerged:

When sulfanilamide was added to whole defibrinated blood so that the concentration was 7 mg. or more per 100 c.c., there was definite bacteriostasis, and in some instances a definite bactericidal effect was demonstrated.

We were unable to demonstrate a bactericidal effect of sulfanilamide when it was added to plasma, although bacteriostasis was present in plasma containing sulfanilamide. This bacteriostasis was less striking in plasma than it was in whole blood.

The samples of blood which showed a bactericidal effect with sulfanilamide were those containing some natural antibodies. It is suggested, therefore, that sulfanilamide may enhance the bactericidal effect of whole blood provided natural antibodies are present.

In the cases in which it was not possible to demonstrate a bactericidal effect, a bacteriostatic effect was shown. When the number of organisms was small, there was almost complete bacteriostasis; when the inoculum was larger, the rate of multiplication was slower and the number of organisms at the end of twenty-four hours was less than in the controls.

It appears that antibodies are important in destroying hemolytic streptococci *in vitro* even in the presence of sulfanilamide.

The principal action of sulfanilamide *in vitro* is to slow the growth of the organism. It has no direct bactericidal effect on any serologic type of hemolytic streptococci.

**LEUKOCYTOSIS, Neutrophilic, in Spinal Fluid Associated With Cerebral Vascular Accidents,** Townsend, S. R., Craig, R. L., and Braunstein, A. L. Arch. Int. Med. 63: 848, 1939.

Six cases of cerebral softening and cerebral hemorrhage in which a great excess of polymorphonuclear leucocytes was found in the spinal fluid are described.

The authors believe that this leucocytosis is due to an aseptic meningeal reaction to adjacent areas of necrosis within the brain.

It is only in cases in which the lesion is close to the surface of the cortex or approaches the wall of the ventricle that leucocytosis occurs, for deeply seated lesions cause no such reactions in the spinal fluid.

The leucocytosis usually begins within several hours after the infarction and gradually disappears within the next five to six days. The number of cells varies between 50 and 3,000 or more per cubic millimeter.

In cases in which the leucocytosis is great there may be stiffness of the neck and a positive Kernig sign, i.e., clinical evidence of meningeal irritation.

In cases in which the vascular lesion does not cause demonstrable focal signs leucocytosis of this type in the spinal fluid may result in an erroneous diagnosis of meningitis.

Other writers have made similar observations, and a brief discussion of the literature is appended.

**PHOSPHATASE, Serum, Influence of Liver on, Schiffman, A., and Winkelman, L.** Arch. Int. Med. 63: 919, 1939.

Serum phosphatase values increase more than tenfold after ligation of a single hepatic duct. No other important changes in the blood result from this procedure.

After the rise that follows obstruction of a single hepatic duct, the values steadily decrease and in thirty to fifty-five days return to normal limits.

Obstruction of the common bile duct in a dog causes a rise in serum phosphatase more than twice that which follows a similar obstruction in other dogs whose livers have been damaged by intravenous administration of arsenic trioxide.

The resultant values of serum phosphatase in hepatic and biliary disorders are dependent on two equally important factors: (a) the degree and extent of biliary obstruction and (b) the functional state of the liver cell.

**TRICHINOSIS, Manifestations of, in the Central Nervous System, Evers, L. B.** Arch. Int. Med. 63: 949, 1939.

A case of trichiniasis which presented a clinical picture of toxic encephalitis and neurorinitis, with subsequent recovery, is reported. Motile larvae were isolated from the spinal fluid after the encephalitis signs had largely disappeared.

Review of the literature reveals that the presence of larvae in the spinal fluid does not always produce clinical manifestations of involvement of the nervous system. Larvae of *T. spiralis* in the spinal fluid have been reported in 24 patients. Of these, 4 were fatal, a mortality rate of 26.6 per cent.

From the reported cases it seems probable that careful examination of the spinal fluid in suspected cases of trichiniasis, with or without symptoms of involvement of the central nervous system, may aid in the diagnosis of this disease.

**HYPERTHERMIA, Therapeutic, Pathological Changes Following, Lichtenstein, L.** Am. J. Path. 15: 363, 1939.

A description is given of the changes observed at autopsy in a case of uncontrollable hyperpyrexia (109° F.) ensuing upon hyperthermia treatment for arthritis of the finger joints. The hyperpyrexia (which developed in the course of the third of a series of treatments) was associated with coma and respiratory failure, and the patient died about thirty-five hours after the fever was initiated. In this case the significant pathologic changes were the following: (1) multiple punctate hemorrhages and necrobiosis in the gray matter of the cerebral cortex; (2) hemorrhage in the left internal capsule; (3) thrombosis of venules and capillaries in the cerebral cortex and internal capsule; (4) cerebral congestion and edema; (5) infarction of kidneys and spleen; (6) marked hepatic degeneration and edema; and (7) pulmonary congestion, hemorrhage, and edema.

The changes found in this case have been correlated with, and discussed in relation to, those in 9 cases previously recorded in the literature. Certain of the changes seen in the case reported here, notably the vascular lesions, have not hitherto been described in connection with fatalities following fever therapy. Specifically there seems to be no previous description of the thrombosis of venules and capillaries in affected portions of the brain, and of the infarcts in kidneys and spleen, apparently due to focal necroses of small arterial branches of these organs.

The principal complications and sequelae of hyperthermia, and especially its effects upon the brain, blood vessels and liver, are indicated. Attention is also drawn to the fact that the reactions to therapeutic hyperthermia are sometimes serious even when they are not fatal.

**LEAD, in Human Tissues, Bagchi, K. N., Ganguly, H. D., and Sirdar, J. N.** Indian J. M. Research 26: 935, 1939.

Lead is normally present in almost all human tissues in variable quantities. Individual variation in the amount of lead is due to difference in the quality and quantity of food ingested. The lead content of normal tissues of the Europeans, as observed by

the European workers, is much higher than that of Indians. The nature of diet and the conditions of living in the West are responsible for this difference.

The dithizone method has been employed for determination of lead in this investigation. It has been proved by the American workers that this method is as good as the spectrographic method, provided the amount of lead to be estimated exceeds 0.001 mg.

Bone, tooth, and hair retain large quantities of lead. The maximum amount is found in hair, especially on the black hair of Indian women. The color of hair appears to depend on its lead content. The skin, in spite of the fact that it is histologically closely related to hair and contains numerous hairs and hair follicles, is very poor in lead.

The ovary is the only organ which has been found free from lead, and thus it differs from testis which contains quite an appreciable amount. The foetal tissues do not show any affinity for lead, although it is believed otherwise.

In cases of abnormal exposure to lead, the liver, stomach, kidney, and lungs show well-marked increase in their lead content, and thus indicate the route through which lead gets into the system.

**CHOLERA, Differential Isolation of *Vibrio Cholerae*, Read, W. D. B. Indian J. M. Research 26: 851, 1939.**

Various methods are described and investigated with a view to the differential isolation of *V. cholerae* from mixtures of cultures, stools, and natural and artificial waters inoculated with *V. cholerae*.

A modification of the bismuth sulfite enrichment medium of Wilson and Blair was the best and enabled the vibrio to be isolated from an inoculum that would just grow only in ordinary broth.

By the use of this medium mannose-fermenting vibrios can be successfully differentiated from nonmannose-fermenting vibrios and from coliform types. Other common water and stool organisms except streptococci are suppressed, but no method of facilitating *V. cholerae* as against manno-se-fermenting inagglutinable vibrios was discovered. The value of the method will depend on whether the mannose-fermenting vibrios found in natural sources can outgrow *V. cholerae* or not. The difficulty due to the growth of total organisms when ordinary peptone water enrichment is employed is overcome.

**CALCIUM, Serum, in Newborn, Denzer, B. S., Reiner, M., and Wiener, S. B. Am. J. Dis. Child. 57: 809, 1939.**

A study of the calcium level in the cord blood and of the blood calcium curve of the neonatal period has been made.

There is a distinct drop in the calcium level of the blood during the first four days of life, with a subsequent slow rise to a point slightly above the average level of later infancy. This depression of the calcium content is not related to race, weight at birth, or neonatal loss of weight.

The inorganic phosphorus content of the blood during the neonatal period is higher than the phosphorus content of the cord blood but shows no definite constant curve.

The protein level of the blood remains constant during the neonatal period and, therefore, cannot affect the level of the available ionizable calcium.

The data derived from a comparison of calcium and phosphorus curves in the neonatal period do not supply an adequate explanation of tetany neonatorum.

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

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### Anemia in Practice\*

THE work of Minot and Murphy in the development of liver therapy in anemia is now a part of medical history and more than suffices to establish the competence of the author to discuss anemia and its treatment.

The outstanding feature of this book is its not only clear and succinct style but also its eminently practical character. While of definite interest and value to the clinical pathologist and hematologist, it may be regarded as a "must" item for the physician's library as a text abounding in clinical implications and applications. There can be no clinical confusion about anemias and their management for the reader of this book.

There are two main sections covering Hypochromic and Normocytic Anemias and Pernicious Anemia. Both are excellent, clear, tersely expressed and yet comprehensive.

This book can be highly recommended.

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### Experimentelle und klinische Ergebnisse mit der Friedmannschen Tuberkulosevaccine†

THE author reports in a book of 155 pages his experiences with Friedmann's vaccine in animal experiments and in clinical observations in human cases of pulmonary tuberculosis. The results are completely negative, and he shows repeatedly the inefficiency of Friedmann's treatment by detailed accounts of each animal experiment and each human case. He reports on 6 rabbits, 50 guinea pigs, and 126 cases in man. He finds that Friedmann's turtle vaccine does not produce an active immunization against tuberculosis in warm-blooded animals. The purpose of the book is to counteract the unjustified recommendation of Friedmann and his followers.

The book does not add anything new to the extensive experience of other investigators on this same subject, and it might well have been much shorter.—*Lotte Kimmelstiel.*

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### Vitamins and Vitamin Deficiencies‡

THOUGH the vitamins are a relatively recent discovery, the literature has reached such astonishing proportions that one who seeks information may find it almost overwhelming.

In this volume, apparently the first of a series, Dr. Harris has presented in a crisp, succinct, and exceedingly interesting manner a historical and introductory account of vitamins B and B<sub>1</sub>.

Dr. Harris is not only admittedly competent to discuss this complex subject with authority but, in addition to a thorough familiarity with the literature, has a distinct flair for developing the interest and even drama of his subject.

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\*Anemia in Practice. Pernicious Anemia. By William P. Murphy, A.B., M.D., Associate in Medicine, Harvard Medical School; Senior Associate in Medicine, Peter Bent Brigham Hospital; Consultant Hematologist, Melrose Hospital, Melrose, Mass. Cloth, 344 pages, 40 figures, 5 colored plates. W. B. Saunders Co., Philadelphia, Pa.

†Experimentelle und klinische Ergebnisse mit der Friedmannschen Tuberkulosevaccine. By E. Haefliger. G. Thieme, Leipzig, 1938.

‡Vitamins and Vitamin Deficiencies. By Leslie J. Harris, Ph.D., D.Sc., F.I.C., Nutritional Laboratory, Medical Research Council and University of Cambridge. Historical. With a Foreword by Sir Frederick Gowland Hopkins, F.R.S. . . . . and fabric, 204 pages, 50 illustrations, \$2.50. P. Blakiston's Son & Co.,

As here told, the history of the discovery of the vitamins is of absorbing interest. After each chapter there is a brief, but meaty, summary in which the essence of the chapter is condensed, followed by a reference list applicable to the matters discussed. Complete subject and author indices are found at the end of the volume.

Those who are interested in the history of the vitamins, in the story of their development, in the methods for their detection and standardization, and in the diseases traceable to their influence—in this volume beriberi, scurvy, and rickets in particular—will find this book excellent. It is well organized, very well written, attractive in format and can be highly recommended.

THIS book needs no introduction and in its present ninth edition presents a survey of medicine in all its varied phases. It has been extensively revised and has been largely rewritten to embody the advances of the last three years.

Not intended as a comprehensive practice, but as a reasonably comprehensive bird's-eye view of newer concepts in the field of clinical medicine, the present edition maintains the standard set by its predecessors upon which their popularity is justly based.

THIS book is complete and thorough in every respect as a text on physical diagnosis for the medical student. It is essentially clinical in character and presents the facts as simply as possible. However, at the same time the subject matter is entirely comprehensive so that it may be used also as an excellent reference by the intern and the practitioner.

In addition to descriptions for routine physical examination, there is a special section devoted to examination of sick children by Norman B. Capon, M.D., F.R.C.P. There are also chapters dealing with operations and instrumental investigations, clinical pathology, and biochemistry, as applicable to clinical medicine.

**BERGEY'S MANUAL** needs no introduction for it has been the standard reference in bacteriologic circles so long that it has become a classic.

This latest edition is greatly increased in size, showing an increase of 368 pages over the edition of 1934 and containing descriptions of 1,335 species. The book, in addition to the addition of new organisms, shows many changes and comprehensive revision.

An introductory section includes a historical survey, the rules of nomenclature, and a discussion of the identification and naming of bacteria.

As usual this is a "must" item for the bacteriologist and laboratory worker.

\*Recent Advances in Medicine. Clinical, Laboratory, Therapeutic. By G. E. Beaumont, M.D., F.R.C.P., D.P.H., Physician to the Middlesex Hospital, etc.; and E. C. Dodds, M.V.O., D.Sc., F.R.C.D., M.D., F.R.C.P., Courtauld Professor of Biochemistry in the University of London, etc. Cloth, ed. 9, 431 pages, 42 illustrations, \$5.00. P. Blakiston's Son & Co., Philadelphia, Pa.

†Symptoms and Signs in Clinical Medicine. E. Noble Chamberlain, M.D., M.Sc., F.R.C.P. Ed. 2, 318 illustrations including 19 in color. William Wood & Co., and Williams & Wilkins Co., Baltimore, Md., 1938.

Wood & Co., and Williams & Wilkins Co., Baltimore, Md., 1938.



### Sex and Internal Secretions\*

PERHAPS few phases of medicine have been the subject of more extensive and intensive investigation within recent years than that included in the studies of internal secretion. Ten years ago, under the guidance of the Division of Medical Sciences National Research Council and with the interest and aid of the Bureau of Social Hygiene, a study was begun on the fundamental problems of sex, the results of which are surveyed in the present volume.

Research problems and their study are always a combination of the abstract and the concrete and lead inevitably to the further problem of their ultimate correlation. To this end surveys of this character which, as it were, take stock of what has been done, are essential in evaluating results and in pointing the way to problems yet to be studied.

This volume presents a cooperative survey of recent advances in research in internal secretions and sex and as such may be taken as a comprehensive and authoritative statement of the problem as it stands today.

The book has five main sections: A. Biological Basis of Sex; B. Physiology of the Sex Glands, Germ Cells and Accessory Organs; C. Biochemistry and Assay of Gonadal Hormones; D. The Hypophysis and the Gonadotropic Hormones of Blood and Urine in Relation to the Reproductive System; and E. Additional Factors in Sex Functions and Endocrine Applications in Man.

The editors, the associate editors, and the list of contributors call the roll of the foremost investigators in this field.

This is a volume of significant importance well deserving of what has been said of its predecessors: "the most authoritative work published in any language on the endocrine aspect of sex physiology."

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### Clinical Bacteriology†

THIS book is essentially practical in its outlook, technical descriptions and details of cultural reactions being briefly discussed and the main emphasis placed upon the application of bacteriologic and immunologic procedures to the diagnosis, prophylaxis, and treatment of disease.

The author's style is exceedingly happy and in many ways reminiscent of Boyd in his *Pathology*. The descriptions of laboratory methods are lucid, the summaries of disease are adequate, and the discussions of treatment are such that clinical applications are at once apparent.

The organization of the book follows customary forms: classification, immunity, hypersensitivity, and general bacteriology following in that order. Theoretical discussions of immunity and hypersensitivity are brief, but their practical applications are treated at some length.

A description of each organism is followed by its cultural and biochemical activities, then by a short description of the diseases to which it has etiologic relation and then by a fairly detailed discussion of diagnosis and treatment. The human pathogens are more fully discussed than animal pathogens and nonpathogens, though all are covered.

Except for the pneumonias, the book is fully up to date. The author does not accept fully the value of serum treatment of pneumonia except in type I and II pneumococcal pneumonias. The value of sulfanilamide compounds in pneumonia is, however, discussed.

As a review of the subject for the physician in practice or in preparation for state board examinations, this book can be highly commended.

\*Sex and Internal Secretions. A Survey of Recent Research. Edited by Edgar Allen. With a Foreword by Robert M. Yerkes. Cloth, ed. 3, 1346 pages, numerous illustrations, \$12.00. Williams & Wilkins Co., Baltimore, Md.

†Clinical Bacteriology. By F. A. Knott, M.D., M.R.C.P., D.P.H., Director Bacteriological Department and Lecturer in Bacteriology, Guy's Hospital. Washable fabric. 426 pages, 12 plates. P. Blakiston's Son & Co., Philadelphia, Pa.

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## CLINICAL AND EXPERIMENTAL

### THE TOXICITY OF VARIOUS IODINE SOLUTIONS\*

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IN 1923 Plummer<sup>1</sup> definitely established the use of iodine in the pre-operative treatment of thyrotoxicosis. He used Lugol's solution, 5 per cent iodine, and 10 per cent potassium iodide, but since then numerous other iodine solutions have been prepared for similar use. We have made a study of the relative toxicity of some of the iodine solutions used clinically, such as Lugol's solution, 10 per cent sodium iodide, 10 per cent potassium iodide, Amend's solution and others, which will be mentioned later. Amend's solution is essentially a mixture of sodium iodide and iodine, the "free" iodine being adsorbed to a protein precipitable with tungstic acid. It was decided to compare the relative toxicity of these solutions by the blood pressure method, the intravenous "emesis" point, and the blood iodine tolerance test, and by administering them to human patients known to be sensitive to iodine.

*Blood Pressure Method.*—Dogs anesthetized with basal ether anesthesia were used. A continuous record of blood pressure and respiration was made. The iodine solutions were injected intravenously during a period of ten to fifteen seconds. The blood pressure was always allowed to return to the control level before the injection of a solution. Doses of each solution equivalent in regard to iodine content were always used. The dogs weighed approximately 10 kg.

The results on the most interesting solutions of iodine are best compared by inspecting the composite curves of the results on 5 dogs in Chart 1. The curves show the average blood pressure depressing effect of 0.1, 0.2, 0.3, and 0.4 gm. of iodine in the various solutions. The composite results on the other solutions are shown in Table I in more detail.

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TABLE I

COMPARATIVE AVERAGE EFFECT OF VARIOUS IODINE SOLUTIONS ON BLOOD PRESSURE\*

SOLUTION	GM. I	MG. PER KG.	AVERAGE B.P. CHANGE IN MM.	REACTION TIME
Amend's	0.1	9-11	3	Immediate reaction returning to normal in 30 sec.
Lugol's	0.1	9-11	16	Immediate reaction returning to normal in 30 sec. to 60 sec.
10% KI	0.1	9-11	13	Immediate reaction returning to normal in 60 sec.
10% NaI	0.1	9-11	2	Immediate reaction returning to normal in 30 sec.
Amend's	0.195	18-22	2	Immediate reaction returning to normal in 10 sec. to 30 sec.
Lugol's	0.195	18-22	22	Immediate reaction returning to normal in 60 sec.
10% KI†	0.195	18-22	9	Immediate reaction returning to normal in 30 sec. to 60 sec.
10% NaI	0.195	18-22	4	Immediate reaction returning to normal in 10 sec.
Amend's	0.390	36-44	12	Gradual slow decrease returning to normal in 2 to 3 min.
Lugol's	0.390	36-44	2 cases—fatal	Immediate reaction
10% NaI	0.390	36-44		Immediate reaction returning to normal in 10 sec.
Amend's	0.47	52	8	Gradual slow decrease returning to normal in 3 min.
Amend's	0.1	3-7	2	Immediate reaction returning to normal in 30 sec.
K Lugol's	0.1	3-7	22	Immediate reaction returning to normal in 1 min. 30 sec.
Na Lugol's	0.1	3-7	14	Immediate reaction returning to normal in 1 min.
Na Lugol's plus 1% gelatin	0.1	3-7	5	Immediate reaction returning to normal in 30 sec.
Amend's	0.2	6-14	2	Immediate reaction returning to normal in 1 min.
K Lugol's	0.2	6-14	57	Immediate reaction returning to normal in 1 min. 30 sec.
Na Lugol's	0.2	6-14	20	Immediate reaction returning to normal in 1 min.
Na Lugol's plus 1% gelatin	0.2	6-14	8	Immediate reaction returning to normal in 45 sec.
Amend's	0.3	12-28	2	Immediate reaction returning to normal in 1 min.
K Lugol's	0.3	12-28	124	Gradual reaction returning to normal in 5 min.
Na Lugol's	0.3	12-28	34	Immediate reaction returning to normal in 1 min. 30 sec.
Na Lugol's plus 1% gelatin	0.3	12-28	22	Immediate reaction returning to normal in 1 min.
Amend's	0.4	24-56	6	Immediate reaction returning to normal in 1 min. (only case—40 mg. per kg.)

\*Five dogs were used ranging from 9 to 11 kg.; ether anesthesia used in all cases.

†In two cases this dosage caused death of the animals.

When 0.1 gm. of iodine was injected in the form of the various solutions, Amend's solution depressed blood pressure four times less than 10 per cent potassium iodide, five times less than Lugol's solution, while 10 per cent sodium iodide had the same effect as Amend's solution.

When 0.2 gm. of iodine was injected, Lugol's solution depressed blood pressure eleven times more than Amend's solution, and 10 per cent sodium iodide two times more. Two dogs died after the 0.2 gm. of 10 per cent potassium iodide.

When 0.4 gm. of iodine as Lugol's solution was injected, it proved fatal in 2 of 5 dogs. Amend's solution and 10 per cent sodium iodide had a relatively slight effect.

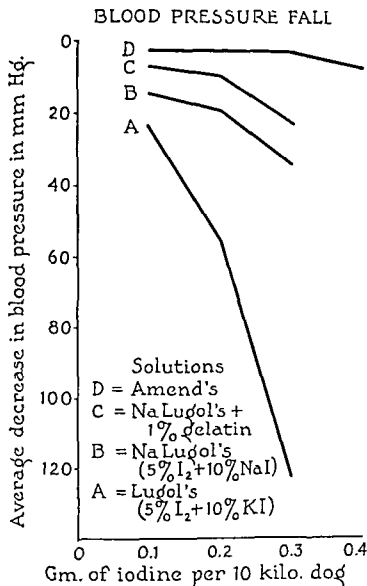


Chart 1.

Since Amend's solution is essentially a mixture of sodium iodide and iodine, the iodine being bound to a proteinlike substance, the toxicity of Amend's solution and a solution of 10 per cent sodium iodide and 5 per cent iodine were compared by the blood pressure method. The sodium-Lugol's solution was prepared and injected in equivalent iodine doses. The composite results may be compared by inspecting curves B and D in Chart 1. In doses of the two solutions of from 0.1 to 0.3 gm. of iodine, Amend's solution is less toxic than sodium-Lugol's solution, although the latter is considerably less toxic than potassium-Lugol's solution. This showed that the proteinlike substance in Amend's solution actually buffers the free iodine.

We then tried the effect of adding proteins to the sodium-Lugol's solution. We used 1 per cent gelatin, 0.5 and 1 per cent egg albumen, and 1 and 2 per cent gastric mucin. All the solutions proved to be less toxic by the blood pressure depressing test than sodium-Lugol's solution alone. The least toxic solution was sodium-Lugol's plus 1 per cent gelatin. The comparative effects of Amend's and sodium-gelatin-Lugol's solution are shown in Chart 1. The results show that protein substances can serve as a buffer for free iodine and reduce the toxicity of iodine solutions, such as sodium-Lugol's solution.

TABLE II

SUBJECT	AMOUNT IODINE 100 MG. I	BLOOD IODINE PER 100 C.C.		URINE IODINE TOTAL PER 24 HR.		% IODINE ELIMINATED IN URINE
		CONTROL	PEAK	CONTROL	POST IODINE	
J	Amend's	1.4	15.5	45.0	763.0	0.7
	Lugol's	5.6	8.4	180.0	785.0	0.7
M	A.	1.4	7.0	53.0	3603.0	3.6
	L.	5.6	8.4	168.0	1220.0	1.2
M	A.	0.70	8.4	20.0	8265.0	8.2
	L.	1.4	9.8	108.0	11426.0	11.4
R	A.	5.6	12.7	322.0	1161.0	1.2
	L.	4.2	11.3	193.0	835.0	0.8
B	A.	2.1	21.1	66.0	3993.0	3.9
	L.	2.1	9.8	94.0	2536.0	2.5
H	A.	2.8	11.3	85.0	584.0	0.5
	L.	2.8	11.3	46.0	695.0	0.7
J	A.	1.4	12.7	124.0	911.0	0.9
	L.	4.2	12.7	183.0	920.0	0.9
A	A.	1.4	8.4	146.0	7718.0	7.7
	L.	2.1	8.4	76.0	4104.0	4.1
Average	A.	2.1	12.1	108.0	3375.0	3.4
	L.	3.3	10.0	131.0	2815.0	2.8
Dog 1	A.	2.1	12.0	14.5	765.0	0.7
	L.	2.1	5.6	9.8	197.0	0.2
Dog 2	A.	2.8	6.4			
	L.	5.6	13.1			
Dog 3	A.	3.5	23.9	11.0	886.0	0.9
	L.	5.6	16.8	13.2	246.0	0.2
Dog 4	A.	2.9	26.0	8.8	322.0	0.3
	L.	5.6	28.1	6.7	152.0	0.2
Average	A.	2.8	17.0	11.4	658.0	0.6
	L.	4.7	15.9	9.9	198.0	0.2

*Emesis Point.*—Experience has taught that dogs with food in their stomachs are quite sensitive in regard to the production of emesis when certain toxic substances are injected intravenously. We used this as a test of the relative toxicity of Amend's and Lugol's solutions when given intravenously to un-anesthetized dogs. The dogs were fed a meal, and the least amount of the two solutions required to cause emesis was determined. Five of 6 fed dogs of approximately the same body weight (10 kg.) vomited after the intravenous injection of 100 mg. of iodine in the form of Lugol's solution. Vomiting did not occur with smaller doses. One hundred milligrams of iodine as Amend's solution had no effect. One hundred and fifty milligrams of Amend's solution caused nausea (salivation), slight retching, but no vomiting, in 6 dogs.

In 2 out of 4 dogs 50 mg. of iodine as Lugol's solution was given by stomach tube and the tube washed with 100 c.c. of water; salivation and apparent nausea, but no vomiting, occurred (stomach empty). When 50 mg. of iodine as Amend's solution were given similarly, salivation and other objective symptoms did not result. This difference can be interpreted as showing that the Lugol's solution irritated the stomach and duodenum more than the Amend's solution, the "free" iodine of which is adsorbed to protein.

*Blood Iodine Curves.*—Iodine is normally present in the blood in an average concentration of 4 gamma per cent.<sup>2</sup> Numerous investigators have shown that for a period of several hours after the ingestion of iodine, the blood iodine rises to a maximum and then falls, producing a blood iodine curve. Accordingly, the blood iodine curves of 8 normal human subjects after the ingestion of equivalent iodine doses of Amend's and Lugol's solutions were determined. The iodine tolerance method of Perkins and his co-workers<sup>3</sup> was used. The results for the human and animal studies are given in Table II. In man, the blood iodine increased from 2 to 3 gamma per cent to 12 gamma per cent, and the peak was reached in the second hour after ingestion. In dogs the blood iodine curves for both Amend's and Lugol's solutions show similar but greater changes. The blood iodine increased from 3 to 5 gamma per cent to 15 gamma per cent. However, when Lugol's solution was taken, the peak in blood iodine occurred in one-half an hour, whereas with Amend's solution it occurred in the second hour. We have no explanation for this phenomenon. The difference between the average control and maximum values for Amend's solution and Lugol's solution is well within the experimental error of the blood iodine method.

The amount of iodine eliminated in the urine of human beings, when either Amend's or Lugol's solution was taken, also shows a close correspondence (Table II). The small amount eliminated can be explained by the fact that the iodine method used gives results which are 8 to 10 per cent lower than those obtained by the earlier methods.

*Rate of Absorption.*—The rate of absorption from the gastrointestinal tract of human subjects was determined by the time of occurrence of the peaks in the blood iodine curves, and also by the time of appearance of iodine in the saliva after receiving 50 mg. of iodine solution by Rhexuss' stomach tube. In all cases, when either Amend's or Lugol's solution was ingested, the iodine was detected in the saliva within fifty to sixty minutes. The salivary glands were not stimulated by any such mechanical means as paraffin chewing, which accounts for the delayed appearance in the saliva.

*Uptake of Iodine by the Thyroid.*—We have followed the method of Van Dyke<sup>5</sup> in this work. Eight dogs, weighing approximately 6 kg. each, were used and anesthetized with nembutal (sodium pentobarbital). After careful exposure of the lobes of the thyroid, a control sample consisting of the lower pole of either the right or left lobe was removed. In all cases it was found that the difference in control iodine content between right and left lobes was insignificant. Then, either Lugol's or Amend's solution in 50 mg. iodine doses was injected into the femoral vein. After thirty minutes a suitable sample of the gland was

removed. The remaining iodine solution was then injected, and after thirty minutes the rest of the thyroid tissue was excised. All samples were weighed fresh, and the iodine concentration determined immediately by Stevens' method.<sup>3</sup> Table III gives the results obtained from analyzing portions of the thyroid gland before and after iodine injection. Although the iodine content per gram of the control lobe was found to be very small, the iodine concentration increased several hundred per cent after injection of an iodine solution. It mattered very little whether Amend's or Lugol's solution was used, since, in either case, the iodine was utilized to approximately the same extent by the thyroid tissue.

TABLE III  
UPTAKE OF VARIOUS IODINE SOLUTIONS BY THE THYROID GLAND

EXPER. NO.	WT. OF DOG (KG.)	50 MG. I INJECTED	WT. OF CONTROL LOBE FRESH (MG.)	GAMMA I PER MG. OF CONTROL LOBE	MG. I PER GM. CONTROL LOBE	WT. OF IODIZED LOBE FRESH (MG.)	GAMMA I PER MG. OF IODIZED LOBE	MG. I PER GM. OF IODIZED LOBE	MG. I INCREASE PER GM. OF GLAND
1	5	Amend's Lugol's	140	0.005	0.005	120 81	0.51 0.50	0.510 0.500	0.505 0.495
2	5	Amend's Lugol's	250	0.0028	0.0028	180 290	0.24 0.14	0.240 0.140	0.237 0.137
3	6	Amend's Lugol's	230	0.003	0.003	670 600	0.08 0.09	0.08 0.09	0.077 0.087
4	6	Amend's Lugol's	130	0.005	0.005	440 580	0.13 0.13	0.13 0.13	0.125 0.125
5	6	Amend's Lugol's	150	0.004	0.004	260 250	0.12 0.12	0.12 0.12	0.116 0.116
6	6	Amend's Lugol's	500	0.0014	0.0014	390 860	0.15 0.10	0.15 0.10	0.14 0.09
7	6	Amend's Lugol's	210	0.003	0.003	420 340	0.10 0.11	0.10 0.11	0.097 0.107
8	6	Amend's Lugol's	135	0.005	0.005	325 505	0.16 0.13	0.16 0.13	0.155 0.125

*Iodine-Sensitive Human Subjects.*—In this study we were able to obtain only 3 persons who manifested hypersensitivity to iodine. Two of these subjects manifested the most usual type of iodine hypersensitivity, namely, sialorrhea. One hundred milligrams of iodine as Amend's solution when given orally resulted in excessive salivation and nausea which lasted for thirty-six hours; 20 mg. of iodine as Lugol's solution gave similar toxic effects.

The third subject was extremely hypersensitive to iodine when taken orally or applied to the skin. When Amend's and Lugol's solutions in equivalent iodine doses were applied to the inner aspect of the forearm, the area painted with Lugol's solution showed a definite irritation the next day, and in three days a marked dermatitis with redness, edema, and small papules. The Amend's solution produced no reaction.

#### DISCUSSION

Essentially there are two groups of iodine solutions: those that contain the sodium cation and those with the potassium cation. That this is an important

factor in determining the toxicity of an iodine solution has been fairly well established. It is pharmacologically known that in large doses the potassium ion is more toxic than the sodium ion. Further, Osborne<sup>4</sup> has reported that when potassium iodide is ingested, an average of 15 per cent of the iodine is present in the plasma in combination with protein and the rest as sodium iodide, there being no evidence that potassium iodide is absorbed as such. It was found that potassium iodide is not as well tolerated as sodium iodide, and that more protein iodide is formed by iodine-hypersensitive patients than in the noniodine-sensitive patients. However, we are not in a position to say that more protein iodide is formed with those iodine solutions containing the potassium ion than those with the sodium ion; this would constitute a major investigation.

In an attempt to decrease the toxicity of iodine solutions containing free iodine, various organic substances have been added which physically combine with the free iodine. Thus, the decreased toxicity of Amend's solution in comparison with other iodine solutions can be explained by the presence of the proteinlike substance which acts as a "buffering agent" for the iodine. However, there is no reason to believe that the free or loosely-linked iodine in Amend's solution is absorbed as such. More likely, the loose linkage between the free iodine and the proteinlike substance would be broken, and the iodine converted primarily into sodium iodide and absorbed as the iodide. That sodium iodide is practically as toxic as Amend's solution and much less toxic than Lugol's solution and potassium iodide is borne out by our blood pressure experiments. However, if Lugol's solution is administered in milk, the milk would buffer the free iodine, thus greatly alleviating the irritating effects of this solution when taken orally. Hence, it may be said, that if Amend's and Lugol's solutions were given in milk, there should be no difference between the doses of iodine required to excite the iodine-sensitive patient. What effect the formation of protein iodide has in reference to iodine sensitivity should be investigated thoroughly. It would also be very interesting to know whether the formation of protein iodide represents the essential feature of the therapeutic value of iodine or the toxic manifestations of iodine, or both.

#### SUMMARY AND CONCLUSIONS

We have investigated the relative toxicity of various iodine solutions in reference to blood pressure depression, to the emesis point when injected intravenously or when taken orally, to the blood iodine curve, and to their effect in iodine-sensitive patients.

The relative toxicity of an iodine solution administered orally, intravenously, or percutaneously, appears to be related to the presence of the sodium or potassium ion in the solution, to the presence of a buffering agent to neutralize free iodine if the latter is present, and possibly to the amount of protein iodide formed during absorption of orally administered iodine.

Amend's solution, which according to our analysis is essentially a solution of sodium iodide containing free iodine adsorbed to a protein, and sodium iodide are less toxic when given intravenously, are less irritating to the stomach when taken orally, and are better tolerated by iodine-sensitive subjects than Lugol's



solution in iodine-equivalent doses. According to the results of the blood iodine curve and the uptake of iodine by the thyroid, Amend's solution and sodium iodide should be as effective as Lugol's solution in the treatment of hyperthyroidism when given orally in iodine-equivalent doses.

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## THE CONCENTRATION OF VITAMIN C IN THE BLOOD DURING AND AFTER PREGNANCY\*

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### I. THEORETICAL PART

THE quantity of ascorbic acid excreted in the urine after a tolerance test of vitamin C is a widely used indicator of vitamin C deficiency in human beings. This method, generally regarded as the best, furnishes information concerning the state of repletion of the vitamin C reserves of the body. It is an open question, however, whether deficiency or absence of reserve vitamin C is an indication of pathologic hypovitaminosis or avitaminosis. In laboratory experiments with guinea pigs, for example, it could be clearly shown that the dose of vitamin C necessary for the maintenance of reserve in a state of repletion is almost ten times as great as the minimum protective dose. Guinea pigs given the minimal dose of vitamin C enjoyed full health and life, even though the vitamin C content of their tissues was minimal. Moreover, it was found that guinea pigs whose vitamin C reserve was brought to a maximum by providing them with a diet rich in vitamin C were just as susceptible to avitaminosis when transferred to a diet deficient in vitamin C as guinea pigs which had not previously been provided with a body store of vitamin C.<sup>1</sup> This finding is highly surprising. The observations are strictly applicable for the present only for the case of guinea pigs, but there is no valid reason for assuming that the relationship in human beings is essentially different. Pending further evidence, it seems fair to conclude that before the repletion test can be correctly used as an indicator of avitaminosis, proof must be advanced that it has a different significance in human beings than in guinea pigs. The experimental data now

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available support the conclusion that a heavy retention of vitamin C in the repletion test is evidence that the body vitamin C reserves are partially or totally exhausted but it is not by any means an indication of hypovitaminosis or avitaminosis.

Despite the low level of the reserve, vitamin C metabolism may proceed along normal lines. Every case of scurvy will, therefore, show a marked retention of fed vitamin C, but not every case of retention is an indication of scurvy or even of "prescurvy." The span between the protective and the storage dose is considerable. In statistical studies based on repletion tests the number of healthy individuals classed as deficient remains an unknown factor.

It has been pointed out that the experiments with guinea pigs show that the previous repletion of the body vitamin reserve does not confer a significant advantage. This does not mean that the minimum dose and no more should be administered, but it does suggest that the feeding of large vitamin C quantities is of doubtful utility. The possibility remains that pharmacologically vitamin C in large doses may be useful as a detoxicant, this aspect is not, however, related to the normal function of vitamin C in the body.

Recent experiments throw some further light on the foregoing findings. It has been shown that the behavior of vitamin C in the urinary excretion is that of a threshold substance.<sup>2</sup> Its serum concentration must be raised from a minimum level of 0.78 mg. per cent or from a medium level of 1.0 mg. per cent to a level of 1.4 mg. per cent before excretion can occur. The latter is the threshold saturation level, but 0.78 mg. per cent is still normal and is by no means an indication of scurvy.

It must further be remembered that the excretion of vitamin C in urine depends on several factors. The excretion is greater and the retention is less, for instance, in acid than in alkaline urine. The vitamin concentration of single urine samples is no longer regarded as significant. Even determinations carried out on a day urine may be misleading.

We believe that the surest method of determining an actual vitamin deficiency in contradistinction to a mere reserve deficiency is provided by assaying the concentration of vitamin C in the blood. It is known that the blood vitamin C level reflects the vitamin C balance of the organism.<sup>3</sup> Symptoms of scurvy are unfailingly accompanied by a low blood concentration of vitamin C. Recovery from the disease is accompanied by a corresponding increase in its blood concentration. Thus in the survey of the food distributed by the social service agencies we are able to show that the vegetable and fruit content of the diet in different districts is correlated with the concentration of vitamin C found in the blood. This latter averaged about 1 mg. per cent in our findings; the minimum was never less than 0.78 mg. per cent.

The available information of the behavior of vitamin C in the blood was found to be deficient in several respects. To fill this lack, some general experiments on vitamin C in blood were carried out before work on clinical aspects was undertaken. Vitamin C (reduced form) was determined in the blood according to the method of Farmer and Abt. In this method, as in most others, Tillman's reagent is used for titration. The validity of the values obtained with

this reagent in urine and other biological fluids has been questioned.<sup>4</sup> It must be shown, therefore, that in the method adopted it is actually only vitamin C which is titrated. It may be noted that Farmer and Abt<sup>3</sup> have shown that in scurvy the reducing values of the blood are very low, but that, nevertheless, a residual reducing ability derived from some source other than vitamin C may be present.

Crystallization of blood vitamin C as an osazone<sup>4</sup> is not practicable. To test the titration procedure a method to be described was therefore proposed. The basis of the method is the fact that many plant juices contain an enzyme which almost specifically catalyzes the oxidation of vitamin C. Press juice of cucumbers, for example, contains this enzyme in high concentration.<sup>5-8</sup>

The effectiveness of enzyme solutions obtained was tested on known ascorbic acid solutions under constant conditions. Boiled press juice was found in control tests to be without action. In preliminary experiments it could also be established that serum was without inhibitory action upon the enzyme. Experiments were then set up as follows: 1 ml. of cucumber juice was added to 3 ml. of serum. As a control, 1 ml. of water or boiled juice was added to 3 ml. of serum. The enzyme was allowed to act for fifteen minutes at room temperature.

TABLE I  
ACTION OF CUCUMBER JUICE ON VITAMIN C IN SERUM AND IN PLASMA

NO.	VITAMIN C IN MG. PER CENT			
	SERUM + CUCUMBER JUICE	SERUM CONTROL	PLASMA + CUCUMBER JUICE	PLASMA CONTROL
1	Trace	0.9	Trace	1.1
2	0.12	0.7	Trace	1.3
3	0.14	1.1	Trace	1.2
4	0	0.6		
5	Trace	1.3		
6	Trace	1.3		
7	0	0.8		
8	0	1.4		
9	0	1.0		

Table I shows that the entire reducing ability of serum to Tillman's reagent is almost invariably abolished by the action of cucumber juice. The residual reducing power found in certain cases was almost completely accounted for by the reduction values found in the blank. It is right to conclude therefore that the method of Farmer and Abt gives a measure of the actual concentration of vitamin C in the serum and is not in practice subject to the interference of reducing agents, other than vitamin C.

A second aspect requiring investigation was the velocity of oxidation of vitamin C in the blood. The rapidity with which vitamin C in the urine and other biological fluids is decomposed and the difficulties caused thereby in the way of precise determination are known. We found that the speed of oxidation in serum is relatively much slower. The serum protein was easily shown to be responsible for the oxidation inhibition. Addition of serum to a pure ascorbic acid solution produced the full inhibitory effect; addition of deproteinized serum produced no inhibition whatsoever. The inhibitory effect observed is a com-

mon property of many colloids and is a result of their ability to bind copper by its conversion into an ineffective complex. Gelatin, agar, and gum arabic inhibit oxidation; pectin, on the other hand, stimulates oxidation.

TABLE II  
INHIBITION OF OXIDATION BY ADDITION OF OXALATE

TIME HOURS	WATER	WATER + OXALATE ADDITION 2%	SERUM	PLASMA + OXALATE ADDITION
Phosphate buffer pH 6.9 Ascorbic acid in mg. per cent				
0	1.60	1.65	0.82	0.82
1	1.25	1.64		
2	0.95	1.64		
3	0.62	1.62	0.43	0.47
24	0	1.46	Trace	Trace

Oxidation is inhibited but not suppressed by serum proteins. The loss in vitamin content of the serum may amount to 30 per cent or more within a period of four hours at room temperature. If it is impossible to carry out the analysis of blood samples immediately, the question whether oxidation of ascorbic acid in the blood can be suppressed by artificial additions therefore assumes practical interest. In controlled experiments it was shown that the addition of oxalate to pure ascorbic acid solutions almost completely inhibits the oxidation process (complex formation). This method would be simplest for blood. It was found, however, that the addition of oxalate to blood, i.e., to tested plasma samples, failed to inhibit oxidation.

TABLE III  
EFFECT OF KCN (5 MG. PER CENT) ON THE OXIDATION OF VITAMIN C  
IN SERUM (PLASMA)

TIME HOURS	VITAMIN C IN MG. PER CENT							
	SERUM	SERUM + KCN	SERUM	SERUM + KCN	SERUM	SERUM + KCN	SERUM	SERUM + KCN
1	1.4	1.4					1.4	1.4
1	1.3	1.3	1.2	1.4	1.0	1.3		
2	1.1	1.2	1.1	1.3	0.7	0.7	1.20	1.23
3	1.0	1.0	1.0	1.0				
4							1.12	1.15
24			0.1	0.3			0.47	0.42
1	1.3	1.3						
2			0.7	0.8				
4			0.4	0.5				
8	0.3	Trace						

PURE ASCORBIC ACID SOLUTION, PHOSPHATE BUFFER pH 7.2

TIME HOURS	VITAMIN C IN MG. PER CENT		IN 1 MG. PER CENT KCN
	AQUEOUS SOLUTION		
0		2.26	2.26
1		1.04	2.24
2		0.51	2.24
24		0	2.00

In pure solutions and in many biological media, hydrocyanic acid, the best known retardant of heavy-metal catalyzed reactions, is generally used as an inhibitor of ascorbic acid oxidation. It seemed promising to test this method

in blood, the more so since this method has already been used for this purpose by several investigators.<sup>9, 10</sup> Careful analysis revealed, however, that suppression of oxidation of vitamin C in serum by addition of potassium cyanide is uncertain. Table III compares the effect of potassium cyanide in pure solutions with the effect in serum.

For several experiments, an inhibition of vitamin C oxidation in native undiluted serum by means of hydrocyanic acid could not be obtained. Recently, Farmer and Abt<sup>11</sup> reported their inability to obtain an inhibition of vitamin C oxidation in serum by means of potassium cyanide. The oxidation process in question which occurs equally well in the presence as in the absence of potassium cyanide is, therefore, not a process catalyzed by heavy metals, but an auto-oxidation.<sup>12</sup> A number of substances known for their ability to form complexes with heavy metals and which inhibit oxidation effectively in pure solutions were without influence on oxidation in serum. Heating to 60° C., it may be noted, accelerated the oxidation.

No practical and easy method of inhibiting the oxidation of vitamin C in stored serum was found. It is essential, therefore, to carry out determination of vitamin C in blood immediately after the sample is drawn. A delay of several hours in carrying out the titration may significantly reduce the vitamin concentration found. If the deproteinized blood cannot be analyzed immediately, the use of metaphosphoric acid as a deproteinization agent is advantageous. Table IV compares the values of vitamin C obtained after different times in blood which has been deproteinized by metaphosphoric acid and Folin-Wu reagent, respectively.

TABLE IV

TIME HOURS	VITAMIN C IN MG. PER CENT							
	HPO <sub>3</sub>	FOLIN- WU	HPO <sub>3</sub>	FOLIN- WU	HPO <sub>3</sub>	FOLIN- WU	HPO <sub>3</sub>	FOLIN- WU
1	1.3	1.1	1.4	1.4				
2	1.2	0.8	1.4	0.8	1.0	0.8	1.3	1.0
3			1.3	0.6				
4	1.1	0.41	1.4	0.4	0.9	0.6	1.0	0.4
24	0.8	0.1	0.9	0	0.7	0.2	0.9	0.1
2	1.4	1.1						
4	1.2	0.5						
24	1.1	0.2						

The experiments show that in brief intervals of storage metaphosphoric filtrates suffer only negligible losses; in Folin-Wu filtrates, on the other hand, oxidation is more rapid. No explanation for the superiority of metaphosphoric acid in this respect can be given.

Attempts were made in controlled experiments to influence the stability of vitamin C in serum by varying the H ion concentration. The effect of pH on the oxidation rate of vitamin C in pure solution, urine, tissue juice, etc., has long been known and used to advantage (e.g., determination in the presence of acetic acid). The pH relationship is, however, essentially different.

Controlled experiments showed that in 4 per cent serum solution the stability of vitamin C is maximal at neutral or weakly alkaline pH values. At increasing acidity, the inhibitory effect of the serum on the rate of oxidation becomes progressively weaker until at pH 5 it is at a minimum and practically

negligible. At this pH the stability of vitamin C in serum is least. Acidification of serum samples is, therefore, a disadvantage and not an advantage in analysis. Since the effect of KCN is much more marked in the acid pH range than in the alkaline range, it may be concluded that at acid pH heavy metal catalysis occurs.<sup>13</sup>

TABLE V

TIME HOURS	ASCORBIC ACID IN MG PER CENT					
	pH = 8		pH = 7.2		pH = 5.8	
	SERUM	CONTROL	SERUM	CONTROL	SERUM	CONTROL
0	2.30	2.30	2.35	2.00	2.40	2.10
1	2.11	1.45	2.22	0.98	1.55	1.16
2	2.10	1.16	2.12	0.62	1.15	0.74
3	2.00	0.57	1.90	0.23	0.72	0.20
24	1.15	0	1.05	0	0	0

TIME HOURS	pH = 8			pH = 5		
	SERUM	SERUM + KCN	BLANK	SERUM	SERUM + KCN	BLANK
0	1.80	1.85	1.46	1.78	1.76	1.48
2	1.75	1.83	0.45	0.35	1.34	0.30
3	1.63	1.78	0.13	0	1.25	0
24	0.59	0.66	0	0	0.31	0

## SUMMARY

It has been shown that the behavior of vitamin C in serum is distinctive. The oxidation of the vitamin is inhibited markedly by the serum proteins.

The stability of the vitamin (i.e., the inhibition of oxidation) is greatest at neutral or weakly alkaline pH. On the acid side, the inhibitory effect diminishes until at pH 5 it is minimal (controlled experiments in 4 per cent serum).

Potassium cyanide and other substances of similar activity do not inhibit the residual auto-oxidation of vitamin C in serum. No method of inhibiting this residual oxidation could be found. The determination of vitamin C in the blood must, therefore, be carried out immediately after the blood is drawn. Within a few hours, losses have already occurred. In this respect, deproteinization by metaphosphoric acid presents an advantage, since vitamin C in metaphosphoric acid filtrates remains intact for several hours, whereas in Folin-Wu filtrates under similar conditions it is unstable.

By the action of ascorbic acid oxidase, the reducing power of serum toward Tillman's reagent is practically reduced to zero. The reducing power of serum toward this reagent, therefore, constitutes a valid measure of its vitamin C content. The action of oxidase is not influenced by serum but heavy metal catalysis is inhibited by serum.

## II. CLINICAL PART

During the period between September 8, 1936 to September 24, 1937; the blood vitamin C content of 322 women was examined. The average concentration of vitamin C was found to be 1.01 mg. per cent.

Table VI shows that the average vitamin C concentration of the blood is highest during pregnancy, and lowest after confinement. The latter values, however, were all obtained during August and September, and since at this time of the year the blood vitamin concentration in women is not yet at its normal level, conclusions based on the figures obtained are necessarily tentative.

TABLE VI  
CONCENTRATION OF VITAMIN C IN BLOOD OF HUMAN FEMALES

DESCRIPTION OF SUBJECT	NUMBER OF CASES	AVERAGE BLOOD VITAMIN C CONCENTRATION IN MG. PER CENT
Nonpregnant	47	0.98
Pregnant	207	1.09
After confinement	62	0.787
Total	322	1.01

In Table VII the vitamin level at different months of the year is indicated. The highest values were obtained between October and February inclusive, i.e., during the citrus season; after this the values gradually decreased. This finding, therefore, constitutes further proof of the close correlation between the diet and the concentration of vitamin C in the blood.

TABLE VII  
CONCENTRATION OF VITAMIN C IN BLOOD DURING DIFFERENT MONTHS OF THE YEAR IN PALESTINE

MONTH	NO. CASES TESTED	AVERAGE BLOOD VITAMIN C CONCENTRATION IN MG. PER CENT
October 1936	16	1.24
November 1936	38	1.12
December 1936	19	1.27
January 1937	16	1.20
February 1937	28	1.21
March 1937	9	1.16
April 1937	16	1.09
May 1937	36	0.95
June 1937	32	1.04
July 1937	47	0.95

Among the 207 pregnant women examined, 7 serious cases of hyperemesis gravidarum were found. Whereas the average vitamin concentration in the blood of pregnant women was found in a total of 207 cases to be 1.09 mg. per cent, the average concentration of vitamin C in the blood of the 7 women suffering from vomitus gravidarum was 0.70 mg. per cent; the deviations from this figure were very small.

TABLE VIII  
CONCENTRATION OF VITAMIN C IN THE BLOOD OF WOMEN DURING HYPEREMESIS GRAVIDARUM

NUMBER OF CASES TESTED	DESCRIPTION	AVERAGE CONCENTRATION OF VITAMIN C IN BLOOD IN MG. PER CENT
207	Pregnant	1.09
7	Cases of hyperemesis gravidarum	0.70

It may be suggested on the basis of experience, that the low concentration of vitamin in these cases is a secondary rather than a primary symptom, and is the result of a vitamin shortage created in the blood by continuous vomiting and deficient nutrition.

Recently the treatment of hyperemesis gravidarum by vitamin C has been suggested. Schmidt,<sup>14</sup> Dicker,<sup>15</sup> and others reported success in the use of large vitamin C doses.

Of the 7 patients we encountered, 4 were treated with vitamin C, only one of whom was successful.

Four of the patients were beneficially affected simply by the different environment provided by hospital life, and ceased to vomit four days after admission to the hospital, even without special treatment. One of these 4 had been treated at her home with large doses of vitamin C but with little success. All 4 showed low blood vitamin concentration, the rates being 0.61, 0.65, 0.70, and 0.88 mg. per cent, respectively.

The data collected seem to justify the following conclusions: (1) Administration of vitamin C is not a specific treatment for hyperemesis gravidarum. (2) The low concentration of vitamin C in the blood is a secondary symptom evoked by undernutrition.<sup>16</sup>

In 80 out of 207 pregnant women observed, the state of health of the teeth and the calcium and phosphorus concentration of the blood were determined.

Stepp, Kühnau, and Schroeder<sup>17</sup> expressed the opinion that vitamin C deficiency is primarily responsible for structural teeth deficiencies.

Gaethgens<sup>18</sup> regarded dental caries a symptom of vitamin C deficiency during pregnancy. The severity of the caries in pregnancy, according to this author, is also influenced by vitamin D and calcium deficiency.

Weibel<sup>19</sup> and Seitz<sup>20</sup> both attributed caries during pregnancy to calcium deficiency.

McIlroy<sup>21</sup> and the American Research Committee<sup>22</sup> arrived at the conclusion that "mineral starvation" is responsible for caries. These authors rejected the possibility that vitamin C deficiency is the cause, since vitamin C failed in their experiments to influence the condition in pregnancy.

Data on the state of health of the teeth in pregnancy are classified in Table IX as good, medium, or bad (cases of caries or pulpitis teeth).<sup>27</sup>

TABLE IX

STATE OF HEALTH OF TEETH	NUMBER OF PATIENTS	VITAMIN C AVG.	P AVG.	CA AVG.	AGE AVG.
Good	17	1.13	3.88	10.39	22.2
Medium	27	1.00	3.65	10.39	21
Bad	36	1.01	3.82	10.51	28.8
Total	80	1.05	3.56	10.45	

The concentration of vitamin C in the blood of women is seen to be independent of the state of teeth. There was likewise no difference with respect to the concentration of phosphorus or calcium in the blood. Consistently higher concentrations of vitamin C were recorded in the blood of pregnant women with healthy teeth, but the increase was so small that no certain conclusions can be based on these findings.

The question whether there is a relationship between gingivitis and vitamin C was of some interest. In the recent literature Stepp, Kühnau, and Schroeder<sup>17</sup> state that the cause of bleeding of the gums is vitamin C deficiency.



Seitz<sup>20</sup> found that in about one-half the pregnant patients he examined, a marked swelling and reddening of the gums and a tendency to bleeding set in after the fifth month.

Gaethgens<sup>18</sup> assumed that vitamin C deficiency during pregnancy manifests itself as latent scurvy. Bleeding of the gums is supposed to be the only symptom of the latent disease.

Of the 207 pregnant patients we examined, 43 cases of bleeding gums in women of different ages were recorded. The concentration of vitamin C in the blood of these patients was in no case far from the average of 1.18 mg. per cent. In 7 of the 43 patients, gingivitis was found to be associated with caries. The values obtained in such cases for blood vitamin C were: 0.67, 0.69, 0.95, 0.97, 1.00, 1.00, and 1.05 mg. per cent, respectively. The average value was 0.90 mg. per cent, and thus somewhat below normal. The number of observations is too small, however, to permit final conclusions.

The foregoing results lead to the conclusion that vitamin C deficiency is not the cause of gingivitis during pregnancy. The values of vitamin C in gingivitis are definitely not below normal.

Attention was directed to the concentration of vitamin C in the milk of human mothers after confinement. Sixty-two women were examined during the period from August to September, 1937. The examination was conducted among the patients of the Rothschild Hadassah Hospital, all of whom received the same nourishment. The average concentration of vitamin C in the milk was found to be 4.62 mg. per cent, a value corresponding closely to that indicated in the literature (Bleyer, Cahnmann, and Schlemmer,<sup>23</sup> Harris and Ray,<sup>24</sup> Wachholder,<sup>25</sup> Ferdinand<sup>26</sup>).

One hundred forty-seven tests of the milk of 62 mothers on the first nine days after confinement were carried out. The results are summarized in Table X. A continuous increase in the vitamin C concentration of the milk from the first day on was noted. The quantity of vitamin C obtained by the infants on successive days after birth could be calculated.

TABLE X

Days after birth	1	2	3	4	5	6	7	8
Concentration of vitamin C in the milk, average value in mg. per cent	2.63	3.48	5.41	6.27	5.55	5.86	5.48	5.52
Quantity of milk in gm.		70	140	210	280	350	420	490
Quantity of vitamin C obtained in milk in mg.		2.436	7.57	13.16	15.54	20.51	23.01	27.0

The question whether a relationship exists between the concentration of vitamin C in the blood and in the milk seemed to be of some importance. To decide this point, the data on the concentration of vitamin C in the blood were divided into two categories: patients with vitamin values below 0.75 mg. per cent, and patients with vitamin values above 0.75 mg. per cent. The results are recorded in Table XI.

TABLE XI

NO. OF CASES	CONCENTRATION OF VITAMIN C IN BLOOD	CONCENTRATION OF VITAMIN C IN MILK
	MG. PER CENT	MG. PER CENT
66	Below 0.75	5.1
77	Above 0.75	5.5

Table XI shows that the concentrations of vitamin C in milk and in blood are not closely correlated. The impression was obtained that the vitamin concentration in the milk is maintained at the normal level even when the concentration in blood is below normal. This unexpected possibility will be tested more closely in experiments to be undertaken. According to statements in the literature, a correlation exists between the concentration of vitamin in the milk and in the urine as measured in the repletion test.

It was established that the age of the mother, the number of children previously born, and the weight of the born child do not influence the concentration of vitamin C in blood or in milk.

#### SUMMARY

The aim of this paper has been to show the importance of the estimation of vitamin C level in the blood for the exact diagnosis of a real vitamin C deficiency.

The concentration of vitamin C in the blood of 322 women (pregnant, post partum, nonpregnant) was measured. The average concentration was found to be 1.01 mg. per cent.

In pregnant women (207 cases) the average blood concentration of vitamin C was 1.09 mg. per cent; in post-partum women (62 cases), the average concentration was 0.79 mg. per cent; in nonpregnant women (47 cases), the average concentration was 0.98 mg. per cent.

In severe cases of hyperemesis gravidarum, the blood concentration of vitamin C was consistently low and averaged 0.7 mg. per cent. The low concentration is believed to be of secondary origin. Administration of vitamin C is therefore not causal therapy.

The concentration of vitamin C in the blood of pregnant women was found to be 1.13 mg. per cent when the teeth were in good condition (17 cases); 1.00 mg. per cent when the teeth were in medium condition (27 cases); and 1.01 mg. per cent when the teeth were in bad condition (36 cases).

The concentration of vitamin C in the blood of pregnant women suffering from gingivitis (43 cases) was normal, rating 1.18 mg per cent.

The blood concentration of vitamin C when gingivitis was complicated by caries (7 cases) was slightly below normal, rating 0.9 mg. per cent.

The concentration of calcium and phosphorus in the blood of pregnant women was found to be independent of the condition of the teeth.

The concentration of vitamin C in human milk within the first nine days after confinement was measured (62 cases and 147 tests). The average concentration was found to be 4.62 mg. per cent.

A continuous increase in the quantity of vitamin C in the milk beginning from the first day after birth was observed.

The quantity of vitamin C excreted in the milk per day was calculated to be 2.4 mg. on the second day, and 27 mg. on the eighth day post partum.

The vitamin C concentrations of milk and blood were not found to be definitely correlated.

The age of the mother, the number of children previously borne, and the weight of the newborn infant do not influence the concentration of vitamin C in the milk and in the blood.

The blood concentration of vitamin C after confinement was below normal (less than 0.75 mg. per cent) in 50 per cent of the patients examined. In certain patients, values as low as 0.32 mg. per cent were encountered.

A survey of the concentration of vitamin C in the blood during different months of the year revealed the existence of a markedly higher average during the citrus season.

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## MAGNESIUM: THE EFFECTS OF INTRAVENOUS INJECTIONS ON THE HUMAN HEART\*

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VERY little work is available concerning the parenteral effects of magnesium on the heart. However, it is not uncommon to run across such statements in the literature that "sudden death following the injection of a magnesium salt . . . is not an uncommon occurrence."<sup>1</sup> The literature has recently been summed up by Miller and Van Dellen, who in animal experiments found deleterious effects by magnesium on the cardiac conduction system.<sup>1</sup> The doses they used, however, were massive. In view of the increasing use of magnesium in the treatment of various types of cardiac disease,<sup>2</sup> and even more so in view of the great importance magnesium has assumed recently as a circulation time agent,<sup>3, 4</sup> we thought it appropos to determine in a systematic study the intravenous effects of therapeutic doses of magnesium on the human heart.

### METHOD

One hundred adult patients were used in this investigation. One group of 34 cases was apparently free of cardiovascular disease; another group of 66 cases presented various forms and degrees of cardiac disease as outlined below. A total of 104 sets of electrocardiographic tracings were taken—69 in the cardiac group and 35 in the noncardiac group.

The three standard leads of the electrocardiogram were taken first. The electrocardiographic circuit was completed again, and simultaneously with the taking of Lead I, 10 c.c. of a warmed 10 per cent aqueous solution of magnesium sulfate were injected into a large antecubital vein of the reclining patient. In accordance with the technique of the magnesium circulation-time test,<sup>4</sup> the first 6 c.c. were injected through a No. 18 gauge needle as rapidly as possible (requir-

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TABLE I  
ILLUSTRATIVE ELECTROCARDIOGRAPHIC EFFECTS OF INTRAVENOUS MAGNESIUM SULFATE INJECTIONS

CASE	NAME	SEX	AGE	BLOOD PRESSURE		TRACING I DEPARTURES FROM NORMAL	TRACING II CHANGES DURING MAGNESIUM INJECTION	TRACING III CHANGES ONE HOUR AFTER INJECTION	REMARKS
				SYST.	DIAST.				
112	A. L.	M	50	104	60	None	T <sub>1</sub> raised (was flat)	QRS <sub>1</sub> decreased amplitude; T <sub>1</sub> raised (was flat); QRS <sub>3</sub> increased amplitude	Noncardiac patient Duodenal ulcer
56	A. F.	F	61	160	78	P waves low; QRS slightly slurred W QRS <sub>3</sub>	QRS <sub>2</sub> moderately increased amplitude; W QRS <sub>3</sub> more marked	None	Arteriosclerotic heart disease
70	M. G.	M	63	126	40	Right axis deviation; QRS slurred and widened to 0.12 second; S <sub>1</sub> pronounced in all tracings; T <sub>1</sub> inverted	Absence of S <sub>2</sub> ; R <sub>2</sub> amplitude markedly increased	None	Acute coronary thrombosis; heart block (2:1 to 3:1) delayed conduction; arteriosclerotic heart disease
172	M. S.	M	67	176	78	QRS slurred; QRS <sub>2</sub> W shaped and negative; T <sub>2-3</sub> inverted; T <sub>1</sub> flat	T <sub>2</sub> flat (higher); QRS <sub>2</sub> became positive and lost the W type	T <sub>2</sub> flat (higher); QRS <sub>2</sub> became positive and lost W	Hypertensive heart disease; angina pectoris
53	B. E.	F	50	230	120	Left axis deviation; QRS slightly slurred; Q <sub>2</sub> deep; T <sub>3</sub> inverted	QRS M shaped and more slurred	None	Hypertensive heart disease
8	M. A.	F	54	240	140	QRS slurred; T <sub>1-2</sub> diphasic; left axis deviation	None	None	Hypertensive heart disease
221	R. N.	M	19	94	60	Complete heart block (11/9/37); auricular rate 88; ventricular rate 30; QRS prolonged to 0.18 and slurred; T <sub>1</sub> inverted	None	QRS markedly decreased amplitude; QRS <sub>3</sub> increased amplitude	Old posterior coronary thrombosis; hypertensive heart disease
							QRS <sub>3</sub> narrower and higher (deeper)	Postscarlatinal complete heart block; three previous sets of tracings showed no changes	

Tracing I—Set of three standard leads taken before injection of magnesium sulfate  
 Tracing II—Set of three standard leads taken during injection of magnesium sulfate  
 Tracing III—Set of three standard leads taken one hour after injection of magnesium sulfate

ing between one and one-half and two seconds), and the balance was injected more slowly. In a few cases 20 c.c. instead of 10 c.c. were injected. No injection required more than ten seconds, and most injections required considerably less. In succession, Leads II and III were then recorded, the total time elapsing from the moment of injection until the completion of Lead III being approximately three minutes. The time was spaced equally between the three leads. One hour later the standard leads were again recorded. Three sets of tracings were thus available for each case: a control set, a set taken during and closely after the injection, and a set one hour after the injection. These three sets are hereafter referred to as tracing I, tracing II, and tracing III, respectively. Space does not permit the publication of all of the available data, but illustrative cases are herein presented.

*Noncardiac Group.*—The group of noncardiac patients comprised 34 cases suffering from a wide assortment of diseases. It included 18 females and 16 males, ranging in age from 19 to 73 years. All were free of cardiovascular disease as ascertained by history, physical examination, and electrocardiographic evidence. Thirty-five sets of tracings were taken.

Ten patients showed electrocardiographic changes. Four patients showed delayed effects (that is, in tracing III); six patients developed changes both during the injection period and one hour later. No typical electrocardiographic pattern was observed. During the injection period Lead I was affected in only one case (Case 209) which showed a slight decrease in amplitude  $T_1$ . In one case (Case 61)  $T_3$  was slightly decreased in amplitude; the reverse held true in another case (Case 112). The other changes consisted in very slight variations in amplitude of  $QRS_2$  and  $QRS_3$ . The development in one case (Case 79) during the injection period of occasional U waves in Lead II, which subsequently disappeared, was interesting.

At no time were changes noted in the P waves, the duration of the P-R interval, or the heart rate. Case 112, Table I, had two sets of tracings, of which one showed no changes, and the other minor T and QRS changes. All other cases showed usually one minor variation.

*Cardiac Group.*—The cardiac group comprised 66 patients, 35 females and 31 males, ranging in age from 16 to 75. Sixty-nine sets of tracings were taken. One patient (Case 221, Table I) had four sets of tracings.

*Arteriosclerotic Heart Disease.*—Twenty-eight patients fell in the arteriosclerotic heart disease group; 9 had coexistent diabetes mellitus, of whom 2 had developed acute anterior coronary thrombosis; 3 had healed posterior coronary thrombosis; 2 had partial heart block (one complicated by an acute coronary thrombosis); one had an acute posterior coronary thrombosis; and 3 had auricular fibrillation. Two cases were complicated by pulmonary emphysema (one with bronchial asthma).

Of 10 patients in the arteriosclerotic heart disease group presenting electrocardiographic changes, only 2 showed changes during the injection period alone; 2 showed changes both during the injection and one hour later, and the balance showed deferred changes only. During the injection period the changes



Fig. 1.—Case 56. *A*, Control before magnesium injection. *B*, Increase in QRS amplitude in Lead II and V QRS Lead III during magnesium injection. *C*, No change one hour after magnesium injection.

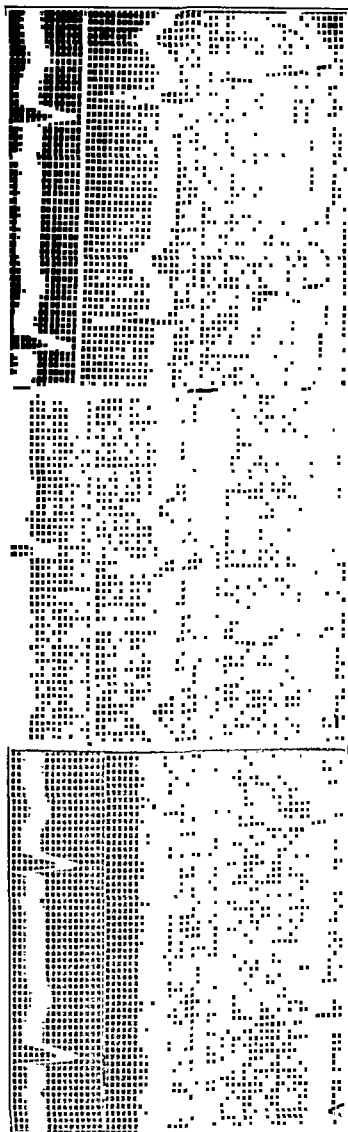


Fig. 2—Case 70. A, Control before magnesium injection. B, Increased  $R_2$  and absent  $S_2$  during magnesium injection. C, Same as B one hour after magnesium injection.



were limited to increase of  $QRS_2$  amplitude and accentuation of W  $QRS_3$  (Case 56, Fig. 1), and increase in amplitude of  $T_3$  (Case 5). Case 70, shown in Fig. 2, presented a marked increase in  $R_2$  amplitude as well as an absence of  $S_2$  in both tracings II and III. One case (Case 3) developed a slight increase in  $T_2$  amplitude in tracings II and III. In the deferred change group (tracing III) were decrease of  $QRS_3$  amplitude (Case 49) and an interesting case (Case 168) in which  $QRS_3$  changed from slurred to W. In one case (Case 13) QRS became lowered in amplitude and W shaped; in another case (Case 64)  $QRS_2$  became lower in amplitude with marked slurring. The left axis deviation present in one case became more marked (Case 72).  $T_2$  became inverted in one case (Case 102).

*Hypertensive Cardiovascular Heart Disease.*—The hypertensive cardiovascular disease group comprised 25 patients; 3 had coexistent diabetes mellitus (one with partial heart block), one had central nervous system syphilis, one hyperthyroidism, one angina pectoris, one had chronic glomerulonephritis, one auricular fibrillation, one a recent anterior coronary thrombosis, one an old posterior coronary thrombosis, and one an acute anterior coronary thrombosis.

Eleven patients in the hypertensive group presented electrocardiographic changes. Two developed changes during the injection period, 6 showed changes as deferred effects and 3 showed changes during the injection period and one hour later. During the injection period one patient (Case 35) developed an increase in amplitude of  $QRS_1$ ; in another case (Case 53, Fig. 3) the  $QRS_2$  complexes became M shaped with increased slurring. One case (No. 31) in which  $QRS_3$  was inverted and W shaped became positive in tracing II and showed a mixture of positive and negative complexes in tracing III. Case 65 showed an increase in  $QRS_2$  amplitude with increased slurring in tracing II, an effect which disappeared in tracing III, only to be replaced by deepening of the  $T_1$  and  $T_2$  waves. Another interesting case (Case 172, Fig. 4) displayed an increase of  $T_2$  amplitude in tracings II and III as well as  $QRS_2$  becoming positive and losing the W. As deferred effects (tracing III) were: QRS showed slightly decreased amplitude in one case (Case 37);  $QRS_2$  decreased and  $QRS_3$  increased in amplitude in one case (Case 8, Fig. 5);  $QRS_1$  amplitude increased and the  $QRS_3$  complex became inverted in another case (Case 69);  $QRS_3$  changed from negative to positive (Case 171);  $QRS_1$  showed slightly increased slurring in one case (Case 212), and the T waves increased slightly in voltage in another case (Case 84).

*Miscellaneous Cases of Cardiac Disease.*—Of a miscellaneous group comprising 8 patients there were 2 with chronic rheumatic heart disease, one with a post-scarlatinal complete heart block, one with a syphilitic dissecting aneurysm of the arch of the aorta, 2 with congenital heart disease (the heart of one was complicated by a subacute bacterial pulmonary arteritis), one with hyperthyroidism and one with a subacute bacterial endocarditis; two patients showed electrocardiographic changes during the injection period and one patient showed changes as a delayed effect. One of the foregoing cases (Case 188) developed inversion of  $T_1$  and  $T_2$ , another (Case 145) an increase of  $R_3$  amplitude during the injection period, and a third (Case 60) a marked decrease of  $R_2$  amplitude as a de-



Fig. 3.—Case 53. A, Control before magnesium injection. B, QRS<sub>2</sub> has become more slurred and M shaped during magnesium injection. C, Showing reversion to control one hour after magnesium injection.

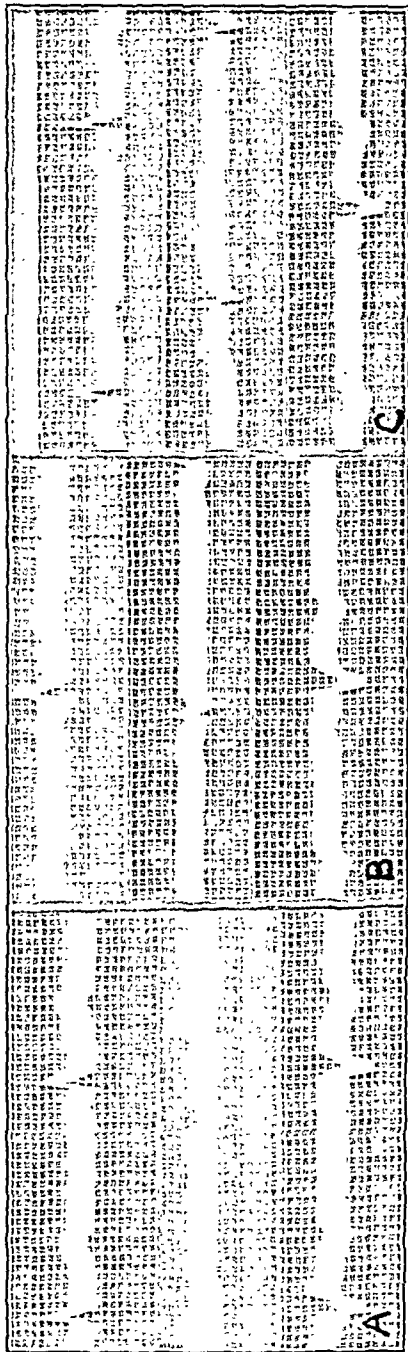


Fig. 1.—Case 172. A, Control before magnesium injection. B, QRS<sub>2</sub> became positive and lost W type. T<sub>2</sub> changed from inverted to upright. C, Same changes as noted in B one hour after injection.

played effect. A case of complete heart block (Case 221)<sup>9</sup> had four sets of tracings, of which the first three showed no changes, and the fourth a narrowing and deepening of the QRS<sub>3</sub> complex one hour after the injection.

*Equivocal Heart Disease.*—In a group of 5 young adults with electrocardiographic changes as the only evidence of cardiac disease, no changes developed during the injection period. One patient (Case 126) developed a sinus arrhythmia and one patient (Case 113) a slightly deeper Q<sub>1</sub> wave in tracing III.

In résumé, the cardiac group as a whole showed slight electrocardiographic changes in 26 of the 66 cases. Only 6 patients were limited to the injection period, 5 to effects both during the injection and subsequently, and 15 patients to the deferred period only. An outstanding feature is that the changes were confined almost exclusively to the T waves and QRS complexes. Even more noteworthy is the fact that during the true injection period (shown only in Lead I of tracing II), only two minor changes were recorded in the entire series. There were no P wave changes, no changes in the P-R interval or in the heart rate. No ventricular premature beats were noted. There were no bradycardias or cases of sinus arrest. Eleven patients who were receiving digitalis at the time of the injections, showed no untoward effects.

#### COMMENT

The striking feature is the complete lack of uniformity of changes wrought upon the heart by magnesium. That magnesium may have a direct effect upon the myocardium of the ventricles is indicated by the slight changes in QRS complexes and T waves occurring in a fairly high percentage of cases. The types of changes produced during the injection period and one hour later are practically the same, whether in the cardiac or noncardiac groups. That these changes are negligible is shown by the fact that Lead I (taken during the injection when the full brunt of the magnesium was borne) showed only one slight T<sub>1</sub> change in the noncardiac group and only one slight QRS<sub>1</sub> and one T<sub>1</sub> change in 2 of the cardiac cases. Moreover, very few patients showed more than one lead change whether during the injection or one hour afterwards. In 2 patients, one a noncardiac person and the other a critically ill complete heart block individual, in whom two sets and four sets of tracings were obtained respectively, it is noteworthy that minor changes were obtained in only one set of tracings in each patient. These results illustrate the inconstancy of magnesium effect. In general, there were as many changes in T waves and QRS complexes in one direction as there were in the opposite. Finally, such changes as did occur were evanescent inasmuch as standard electrocardiograms, taken in the vast majority of the patients within a few days after the injection, showed all magnesium effects to have vanished entirely. Consequently, we must conclude that the rapid intravenous injection of 10 per cent magnesium sulfate exerts no deleterious action on the heart.

It is interesting to compare these results with the cardiac effects induced by the intravenous use of 20 per cent calcium gluconate, a substance that has come into wide use recently as a circulation-time reagent. In normal individuals

calcium produces flattening or inversion of the T waves in 92 per cent of cases, flattening or inversion of the P waves in 54 per cent, and a marked bradycardia in 67 per cent.<sup>5</sup> No systematic studies of the cardiac effects of intravenous calcium injections in cardiac patients are available, so that we have no basis for direct comparison of the effects of magnesium and calcium in cardiac disease. That calcium injections clinically may lead to catastrophes in both normal and diseased hearts is now well substantiated.<sup>5-8</sup>

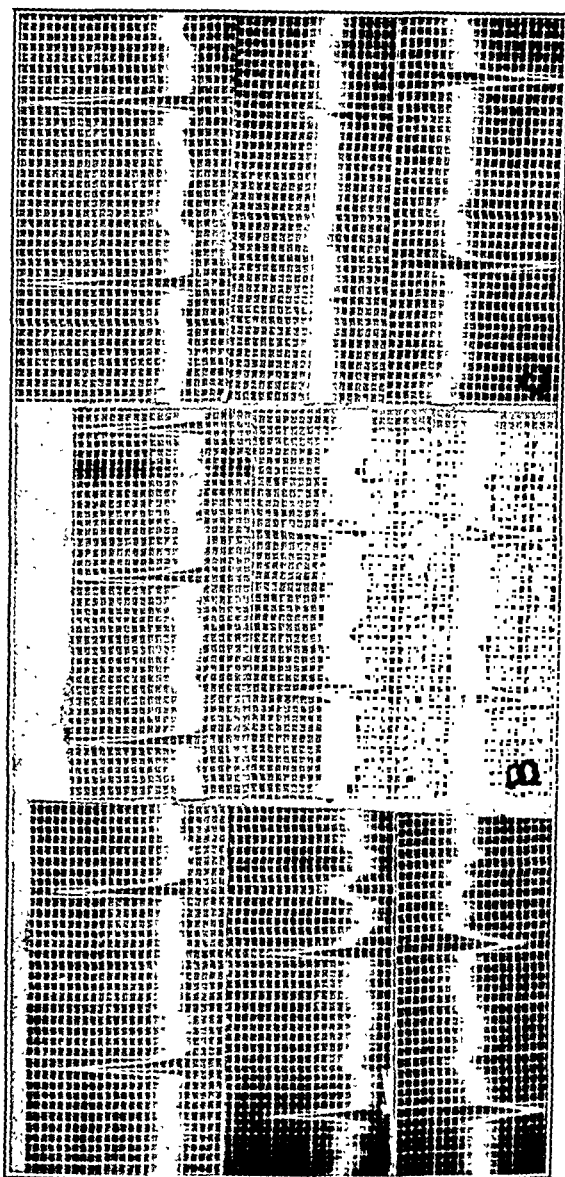


Fig. 5.—Case 8. A, Control before magnesium injection. B, QRS<sub>2</sub> lower voltage and QRS<sub>3</sub> increased voltage during magnesium injection. C, QRS<sub>2</sub> depressed and QRS<sub>3</sub> increased one hour after magnesium injection.

#### SUMMARY

1. The effects of the rapid intravenous injection of 10 c.c. of 10 per cent magnesium sulfate on the human heart were recorded electrocardiographically in 100 patients. The three standard leads of the electrocardiogram were re-

corded prior to injection, simultaneously with the injection, and one hour later. Thirty-five sets of tracings were taken in 34 noncardiac patients and 69 sets in 66 cardiac patients.

2. The cardiac group included 28 persons with arteriosclerotic heart disease, 25 persons with hypertensive heart disease, 5 persons with only electrocardiographic evidence of cardiac disease, and a miscellaneous group of 8 cases comprising 2 persons with chronic rheumatic heart disease, one with postscarlatinal complete heart block, one with syphilitic dissecting aneurysm of the aortic arch, two with congenital heart disease (one complicated by a subacute bacterial pulmonary arteritis), one with subacute bacterial endocarditis, and one with hyperthyroidism.

3. In the noncardiac group 6 patients presented minor T and QRS changes (usually in one lead) both during the injection period and subsequently; 4 persons showed similar changes one hour after injection.

4. In the cardiac group similar changes were exhibited by 6 persons during the injection period, by 15 persons one hour after injection, and by 5 persons both during the injection period and one hour later.

5. A discussion of the changes produced leads to the conclusion that intravenous magnesium injections exert no deleterious effect on the human heart. The coincidental administration of digitalis yields no untoward effects.

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1321 SPRUCE STREET

# CHOLESTEROLYSIS IN THE BLOOD PLASMA OF INDIVIDUALS WITH MENTAL DISORDERS\*

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IN A SERIES of papers it has been demonstrated by one of us (Schube<sup>1-3</sup>) that in abnormal mental states as a group and in its various subdivisions there are certain alterations in cholesterol metabolism which are reflected in the whole blood cholesterol. In additional papers<sup>4, 5</sup> there were also reported studies on cholesterolysis in normal man. In them certain facts were brought out relative to the stability of cholesterol in blood plasma. In view of the cholesterol values in abnormal mental states and of our observations on cholesterolysis in normal man, we feel it pertinent to present a study on cholesterolysis in the blood plasma of individuals with abnormal mental states in the hope of further clarifying the problem of cholesterol metabolism.

## METHOD

The individuals used for this work were all normal physically, but sufficiently mentally ill to necessitate their institutionalization. All were males, females being excluded because of the possible complicating factor, catamenia. The blood was drawn under sterile conditions after a fast of fifteen hours. It was divided into two parts, and an equal quantity placed in each of two sterile oxalate tubes. It was taken immediately to the laboratory where the sample was centrifuged at 30,000 r.p.m. for fifteen minutes, the plasma removed, and the first cholesterol estimation started within a half hour after the withdrawal of the blood. One tube of blood was kept at room temperature (21° to 24° C.) and the other at icebox temperature (8° C.). Subsequent cholesterol estimations were made on samples of blood drawn from these two specimens at one-, two-, four-, six-, eight-, and twenty-four-hour intervals. The method of determination of the cholesterol was adapted from Bloor,<sup>6</sup> where 1 c.c. of plasma was added slowly with shaking to approximately 40 c.c. of an alcohol-ether mixture (3:1) in a 50 c.c. volumetric flask. This was brought to the boiling point on a water bath, allowed to cool to room temperature, diluted to the 50 c.c. mark, and filtered into an Erlenmeyer flask through fat-free filter paper, the funnel being constantly covered to prevent evaporation. Twenty cubic centimeters of this filtrate were evaporated just to dryness at a temperature of 60° to 70° C. The residue was extracted three times with hot chloroform, using first 5 c.c., then 3 c.c., then 2 c.c. of chloroform, and transferring each portion to a 10 c.c. graduated, stoppered cylinder. The extractions were made up to 5 c.c. with

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TABLE I

SHOWING DETAILED VARIATIONS IN CHOLESTEROL VALUES AS MEASURED IN MG. PER 100 C.C. PLASMA IN EACH INDIVIDUAL FOR EACH TIME PERIOD, AND THE GAIN OR LOSS FOR EIGHT- AND TWENTY-FOUR-HOUR PERIODS

CASE	TIME IN HOURS							GAIN OR LOSS	
	$\frac{1}{2}$	1	2	4	6	8	24	8 HR.	24 HR.
<i>Icebox Temperature Group</i>									
1	129.5	128.3	122.5	122.0	122.0	119.6	121.4	- 9.9	- 8.1
2	132.5	136.3	133.7	133.7	132.5	126.3	122.0	- 6.2	-10.2
3	156.2	156.2	166.2	151.2	161.2	155.0	151.2	- 1.2	- 5.0
4	168.7	171.2	186.2	153.7	180.0	180.0	180.0	+ 11.3	+11.3
5	120.1	123.8	120.1	121.0	120.1	122.5	109.6	+ 2.4	-10.5
6	181.2	181.2	182.5	181.2	176.2	182.5	173.7	+ 1.3	- 7.5
7	200.0	200.0	196.2	200.0	200.0	192.5	185.0	- 7.5	-15.0
8	211.5	213.7	215.0	213.7	213.0	205.0	200.0	- 6.5	-11.5
9	160.2	158.7	165.0	165.0	156.0	153.7	147.6	- 6.5	-12.6
10	130.2	130.2	129.5	131.6	131.2	123.7	118.7	- 6.5	-11.5
11	136.2	133.7	133.7	133.7	133.7	147.5	147.5	+ 11.3	+11.3
12	148.7	147.5	150.0	147.5	147.5	167.5	188.7	+ 18.8	+40.0
13	165.0	165.0	161.2	142.5	166.2	165.0	151.2	$\pm$ 0	-13.8
14	187.5	187.5	186.2	188.7	185.0	186.2	165.0	- 1.3	-22.5
15	123.7	122.5	123.7	121.7	122.5	118.7	116.2	- 5.0	- 7.5
16	188.7	187.5	186.2	187.5	188.7	192.5	191.2	+ 3.8	+ 2.5
17	156.2	156.2	170.0	172.5	170.0	168.7	168.7	+ 12.5	+12.5
18	203.7	205.0	240.0	245.0	245.0	250.0	245.0	+ 46.3	+41.3
19	165.0	165.0	165.0	163.7	165.0	165.0	163.7	$\pm$ 0	- 1.3
20	200.0	200.0	198.7	200.0	200.0	245.0	255.0	+ 45.0	+55.0
21	161.2	161.2	160.0	163.7	161.2	155.0	148.7	- 6.2	-12.5
22	192.5	195.0	193.7	192.5	192.5	190.0	177.5	- 2.5	-15.0
23	188.5	188.5	187.5	185.0	188.7	198.7	200.0	+ 10.2	+11.5
24	196.2	196.2	211.2	205.0	206.2	205.0	217.5	+ 8.8	+21.3
25	151.2	152.5	152.5	153.7	155.0	151.2	161.2	$\pm$ 0	+10.0
26	185.0	185.0	185.0	186.7	185.0	185.0	183.0	$\pm$ 0	- 2.0
Total	4339.4	4347.9	4421.5	4361.5	4404.3	4451.8	4389.2	+112.4	+50.2
Avg.	166.9	167.2	170.5	167.7	169.3	171.2	168.8	+ 4.3	+ 1.9

*Room Temperature Group*

1	129.5	126.0	118.3	115.3	123.1	120.1	120.7	- 9.4	- 8.8
2	132.5	131.2	135.5	133.7	132.5	126.3	122.0	- 6.2	-10.5
3	156.2	155.0	155.0	156.2	166.2	160.0	147.5	+ 3.8	- 8.7
4	168.7	168.7	180.0	156.2	153.7	180.0	180.0	+ 11.3	+11.3
5	120.1	124.5	121.3	124.5	124.5	130.0	113.1	+ 9.9	- 7.0
6	181.2	181.2	186.2	187.5	193.7	178.7	167.2	- 2.5	-14.0
7	200.0	200.0	200.0	200.0	201.7	203.7	187.5	+ 3.7	-12.5
8	211.5	213.7	213.7	213.7	213.7	205.0	200.0	- 6.5	-11.5
9	160.2	160.2	160.2	158.7	160.2	150.0	148.7	- 10.2	-11.5
10	130.2	130.2	127.5	131.6	128.7	127.5	116.2	- 2.7	-14.0
11	136.2	135.0	132.5	140.0	135.0	147.5	148.7	+ 11.3	+12.5
12	148.7	150.0	150.0	150.0	150.0	163.7	165.0	+ 15.0	+16.3
13	165.0	165.0	166.2	166.2	161.2	165.0	153.7	$\pm$ 0	-11.3
14	187.5	187.5	187.5	187.5	187.5	165.0	166.2	- 22.5	-21.3
15	123.7	123.7	123.7	123.7	122.5	118.7	116.2	- 5.0	- 7.5
16	188.7	192.5	188.7	188.7	188.7	188.7	188.7	$\pm$ 0	$\pm$ 0
17	156.2	156.2	170.0	168.7	168.0	167.5	168.7	+ 11.3	+12.5
18	203.7	203.7	240.0	240.0	240.0	250.0	250.0	+ 46.3	+46.3
19	165.0	165.0	165.0	165.0	165.0	200.0	200.0	+ 35.0	+35.0
20	200.0	198.7	201.2	203.7	201.2	255.0	271.2	+ 55.0	+71.2
21	161.2	160.0	161.2	165.0	161.2	155.0	142.5	- 6.2	-18.7
22	192.5	191.2	192.5	190.0	190.0	192.5	178.7	$\pm$ 0	-13.8
23	188.7	188.7	187.5	187.5	188.7	198.7	196.2	+ 10.0	+ 7.5
24	196.2	196.2	200.0	205.0	206.2	208.7	218.7	+ 12.5	+22.5
25	151.2	150.0	152.5	153.7	155.0	160.0	162.5	+ 8.8	+11.3
26	185.0	185.0	185.0	185.0	185.0	183.7	183.0	- 1.3	- 2.0
Total	4339.6	4340.0	4401.2	4397.1	4403.2	4501.0	4412.9	+161.4	+73.3
Avg.	166.9	166.9	169.2	169.1	169.3	173.1	169.7	+ 6.2	+ 2.819



TABLE II

SHOWING RANGE OF GROUP VALUES AND AVERAGE OF GROUP VALUES FOR EACH TIME PERIOD THE CHOLESTEROL WAS MEASURED, AND AVERAGE GAIN OR LOSS AND RANGE OF GAINS OR LOSSES FOR ENTIRE GROUP FOR EIGHT- AND TWENTY-FOUR-HOUR TIME PERIODS

TIME (HOURS)	RANGE	AVERAGE	AVERAGE GAIN OR LOSS	RANGE GAIN OR LOSS
<i>Icebox Temperature Group</i>				
$\frac{1}{2}$	120-211	166.9		
1	122-213	167.2		
2	120-240	170.0		
4	121-245	167.7		
6	120-245	169.3		
8	118-250	171.2	+4.7	+46.3 to -9.9
24	109-255	168.8	+1.9	+41.3 to -22.5
<i>Room Temperature Group</i>				
$\frac{1}{2}$	120-211	166.9		
1	123-213	166.9		
2	118-240	169.2		
4	115-240	169.1		
6	122-240	169.3		
8	118-255	173.1	+6.2	+55.0 to -22.5
24	113-271	169.7	+2.8	+71.2 to -21.3

chloroform. In a similar cylinder 5 c.c. of the chloroform-cholesterol standard containing 0.1 mg. of cholesterol was pipetted. To both standard and unknown there was added 2.0 c.c. of acetic anhydride and 0.1 c.c. of concentrated sulfuric acid. Each cylinder was stoppered and mixed and permitted to stand in the light of the colorimeter for twenty-five minutes. The resulting yellow-green colors were compared in the colorimeter, and the cholesterol per 100 c.c. of plasma was calculated from the formula:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 125 = \text{mg. cholesterol per 100 c.c. of plasma.}$$

Each estimation was done in duplicate and the average value recorded in each instance. The error of the method was considered to be  $\pm 5$  mg.

The normal controls were the 21 cholesterolysis studies on normal individuals previously reported.

## RESULTS

The total number of men in the study was 26. Their age range was from 21 to 33 years.

Table I shows the detailed cholesterol values in each individual for each time period as well as eight- and twenty-four-hour gain or loss.

Table II shows the range of gain or loss and the average value of cholesterol for the entire group for each time period.

From these tables it can be seen that the nine conclusions drawn for the normal group<sup>4</sup> alone equally hold true for the mentally abnormal group alone; but when the normal group is compared with the mentally abnormal group, the following differences are noted:

1. The average rate of change of blood cholesterol at room temperature and icebox temperature of the mentally abnormal group and of the normal group is experimentally identical.

2. At the end of eight and twenty-four hours the average cholesterol change of normal group is less than that of mentally ill group both at room temperature and at icebox temperature, although the difference is very small.

3. At the end of eight and twenty-four hours the range of gains for the icebox group is practically the same, but the range of losses is markedly less in the mentally abnormal group than in the mentally normal group. In the room temperature group the range of eight-hour gain is definitely greater, but the difference in the losses is slight; in the twenty-four-hour period the range of gain is definitely greater and the range of loss definitely less.

4. The curve of average cholesterol values of the abnormal group parallels that of the normal group throughout the twenty-four-hour period, but is always significantly below it, both in the icebox group and in the room temperature group. This is illustrated in Chart 1.

5. The range of gains or losses for the eight- and twenty-four-hour periods shows a significant shift in a tendency for the abnormal group to show gains over losses whereas this is not so for the normal group. This is illustrated in Chart 2.

#### DISCUSSION

In an earlier paper on cholesterolysis we pointed out that the problem of the rate of change of cholesterol in a specimen of blood is undoubtedly a complicated one, the result depending entirely upon the direction of equilibrium established by the involved factors: (a) the attempt of a cholesterol esterase to change free cholesterol to a combined form; (b) simple dissolution of cholesterol molecules by hydrolysis; (c) the types of anticoagulant used; and (d) the rate at which the cholesterol aggregates are able to free themselves from the meshes of protein molecules. These factors were thoroughly discussed at that time and to date our work does not present any contradictory material in this respect. The factor (d) then, as now, was the most intriguing, and there are additional studies relating to it which are worthy of presentation. Bellis and Scott<sup>7</sup> and Lehman and Scott,<sup>8</sup> while working with blood, reported a protein shift from cells to plasma following the addition of isosmotic dextrose, sodium chloride, calcium chloride, or barium chloride to oxalated or defibrinated blood *in vitro*. This shift caused an increase in the plasma-protein concentration which was proportional to the degree of dilution. The protein chiefly affected was albumin. Sandor<sup>9</sup> noticed that of the proteins of the blood the albumin fraction frequently contained lipids, of which one was cholesterol, and that the amount of lipid bound to the albumin was fairly constant regardless of the species or the individuals within it. On the other hand, he did not find this to be true of the globulin fraction, for here he found that the quantity of lipid bound to the globulin varied widely in different species and in different individuals within the same species. Moreover, he found that strikingly specific variations existed in the strength of the protein-lipoid bonds, for different alcohol concentrations were required to attain the maximum extraction of serum lipids. He suggested that lipids and proteins are linked through accessory valences rather than enmeshment. Went and Kúthy,<sup>10</sup> in their

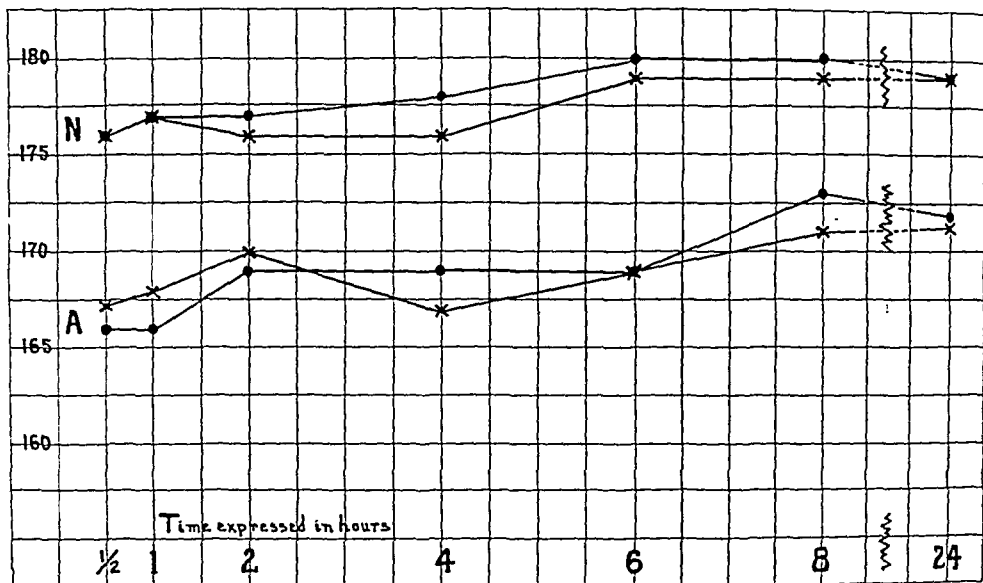


Chart 1.

A=Mentally ill group.

N=Normal control group.

●=Average room temperature values.

X=Average icebox temperature values.

Vertical figures=Milligrams cholesterol per 100 c.c. blood plasma.

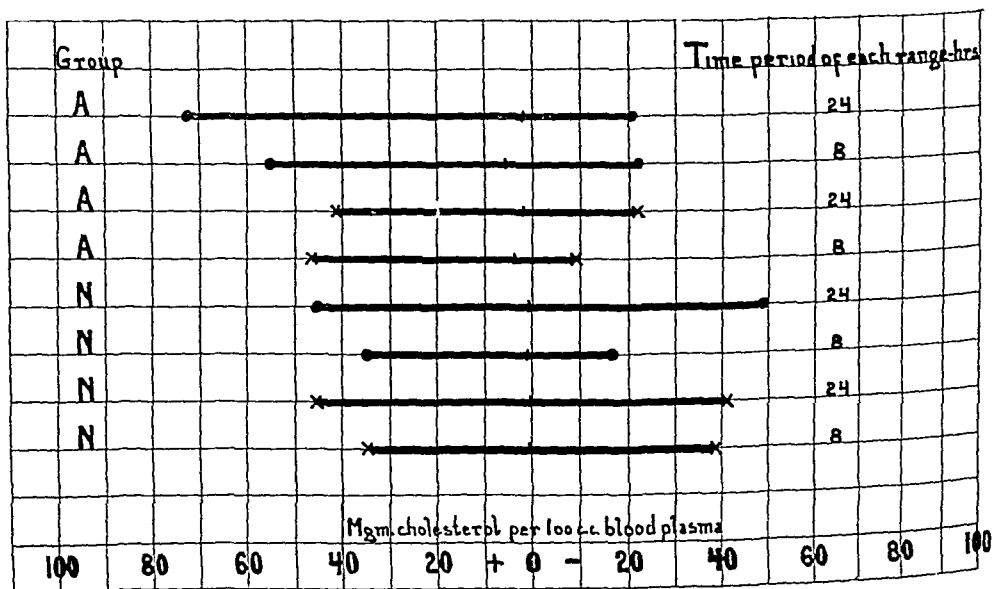


Chart 2.

A=Mentally ill group.

N=Normal control group.

●=Range room temperature values.

X=Range icebox temperature values.

studies of lipoid-protein complexes, found that these were easily formed by the addition of emulsified lecithin to native serum, but not to sera previously treated with alcohol-ether mixture. Süllmann and Verzar,<sup>11</sup> using lipemic blood, found that the lipids would diffuse through membranes in considerable amounts if they used membranes which were at least permeable to Congo red (pore diameter calculated to be  $18 \text{ by } 10^{-8} \text{ cm.}$ ). They discussed the possibility that unsplit protein-lipid complexes might be able to go through capillary walls, at least under special conditions, and feel that this is substantiated by the rough parallelism which appears to exist between the lipid and protein contents of some pathologic fluids. In confirmation of this Bruger<sup>12</sup> reported that in the course of the ultrafiltration of pleural and ascitic transudates, the cholesterol and proteins are concentrated to about the same extent in the ultrafiltration residue. He feels, though, that since cholesterol, but not the proteins, is adsorbed by a Berkefeld candle or by kieselguhr, the bond between the two substances must be a very weak one. Bang<sup>13</sup> long ago first noticed that the precipitation of globulin in the blood carried down with it a portion of the cholesterol, and he suggested at that time that the cholesterol was combined with the euglobulin fraction of the blood protein. Handovsky and Lohmann<sup>14</sup> carried this work farther and have reported that the higher the euglobulin in the blood the less the cholesterol that could be shaken out with ether. They found that 25 per cent of the cholesterol in ox serum was bound with the globulin. Gardner and Gainsborough,<sup>15</sup> likewise studying lipoid-protein complexes, reported that the percentage of the total plasma sterol retained in the proteins was 16.4; that euglobulin retained 13.8 per cent; pseudoglobulin 1.03 per cent; and albumin 1.5 per cent. Theorell,<sup>16</sup> while discussing the physical-chemical aspects of the blood lipids, stated that he felt the cholesterol to be held in combination not only with globulin but also with fibrinogen, and that at the isoelectric point of globulin all of the cholesterol can be extracted with ether; that fibrinogen holds the cholesterol firmly except at pH 6. Machebœuf,<sup>17</sup> while separating compounds from the blood plasma containing both proteins and fatty substances, found that he was able to obtain an albumin fraction very rich in lecithin and cholesterol esters and which was also soluble in neutral or alkaline water to a clear solution containing up to 5 per cent.

These studies establish the following facts relative to factor (d) in the stability of cholesterol in blood plasma: (1) There is a state of protein equilibrium in the blood which can be upset in either direction by interference with the electrolyte balance of the blood. (2) There is a certain quantity of cholesterol adherent to the protein molecule. (3) The largest percentage of cholesterol adherent to the protein is adherent to the euglobulin fraction. (4) The bonds between the cholesterol and the euglobulin are fragile. (5) This bond may be of the nature of enmeshment, accessory valences, or adsorption. It can readily be seen from this that the presence or absence or direction of cholesterolysis in the blood plasma can, and probably does, depend upon the protein molecules therein.

The material evaluated here makes it only too clear that there are at work in the blood plasma of man some such complicated factors as the foregoing,

factors which make group evaluation of cholesterolysis impracticable and which in themselves insist upon individual attention. It is impossible to attribute such individual fluctuations as those observed to any simple variable such as age, sex, weight, or body type, for analysis in this respect only leads to a confusion of the issue. From a metabolic point of view, however, our results are interesting for two reasons: (1) the changes which occur in the plasma cholesterol of individuals with abnormal mental states are practically identical in nature with those of normal individuals, and (2) these changes always average upon a lower metabolic plane. Whether or not these facts are of any significance in mental disorders, it is difficult to state; that they conform with much other physical-chemical data which have accumulated relative to metabolism in neuropsychiatric cases is unquestionably true, for there is a definite body of literature indicating that in mental disorders, and particularly in dementia praecox, a lowered metabolic state exists and that this state closely parallels on a lower plane that of the normal individual. The reasons for this are, unfortunately, unknown, but they are undoubtedly based upon the physical-chemical limitations of the individual cells composing the body, the abnormal metabolism, as the abnormal mental symptomatology, being merely an indicator of abnormal cell function, the reasons for which have to be left to future research. The part which cholesterol plays in this metabolism is not at all clear, for all of our knowledge concerning its function in the metabolism of the individual is indirect and not at all conclusive. That it plays an important part is unquestioned; just what that part is must be left to future research.

#### SUMMARY

A study of the rate of change of total cholesterol in the blood plasma of mentally ill persons over a period of twenty-four hours is presented. Changes do occur in positive and negative directions which are similar to those of normal individuals but on a lower plane. It is very probable that these are not significant for the group, but they may be for the individual case.

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## VITAMIN A DEFICIENCY: ITS PREVALENCE AND IMPORTANCE AS SHOWN BY A NEW TEST\*

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THE importance of vitamin A in human nutrition is not settled. Overardent claims by some have been met with protests by others. The only specific disease commonly associated with vitamin A, xerophthalmia, is so rarely seen that some experts doubt the likelihood of a vitamin A deficiency being common. Recently instruments for testing subnormal dark adaptation have been used as indicators of vitamin A deficiency, but these have been criticised from a clinical viewpoint.

This paper presents (1) details of a new rapid visual test; (2) evidence of the test's relation to vitamin A; (3) evidence of the prevalence and clinical significance of vitamin A deficiency.

### I. A RAPID VISUAL TEST

It is supposed that the recovery of vision after blinding by a bright light depends on a reformation of the retinal visual purple from vitamin A. Reviews connecting this process with night blindness, dark adaptation, visual purple, etc., by Adams,<sup>1</sup> Tansley,<sup>2</sup> Hecht,<sup>3</sup> Jeghers,<sup>4</sup> and Booher,<sup>5</sup> may be consulted. Various instruments for testing dark adaptation have been used, including Aubert's glowing wire of 1865,<sup>6</sup> the Birch-Hirschfeld photometer,<sup>7</sup> and various recent instruments by Tschernig,<sup>8</sup> Edmund,<sup>9</sup> Feldman,<sup>10</sup> Ferree and Rand,<sup>11</sup> and Jeans and Zentmire,<sup>12</sup> who call their instrument a "biophotometer."

The biophotometer, being available commercially, has dominated the recent reports in this field, and with the "visual adaptometer" of Hecht,<sup>13</sup> and the "adaptometer" of Feldman,<sup>23</sup> seems to be the chief instrument being used. These instruments are complicated in construction, seem to require practiced

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subjects for best results, and above all, require ten minutes or more for one test. Furthermore, severe criticisms, especially regarding the relation of these tests to vitamin A deficiency, have been advanced by Snelling,<sup>14</sup> Palmer and Blumberg,<sup>15</sup> Gridgeman and Wilkinson,<sup>16</sup> Isaacs, Jung, and Ivy.<sup>17</sup>

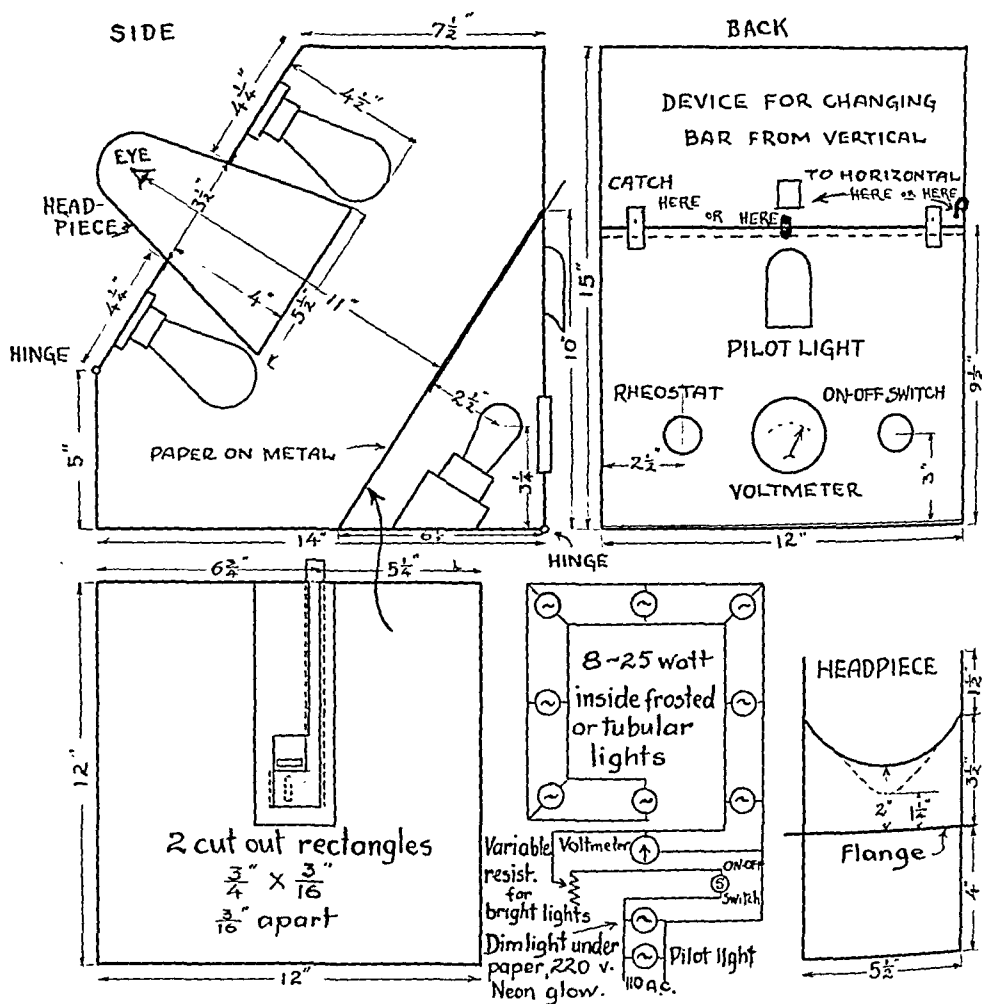


Fig. 1.—Drawings for one model of the apparatus described.

*Description of Apparatus.*—The instrument here outlined consists of a black metal (or wooden) box, about 1 cubic foot in volume. Protruding from the front is a headpiece, and on the back are various instruments, as seen in Fig. 1. Inside the box are: (1) A source of bright white light for bleaching visual purple in the eyes, coming from either one or several bulbs. (2) A surface for reflecting this bright light into the eyes, while looking into the headpiece, such that 50 foot-candles is the intensity at the eyes; the bright light is connected to a voltmeter and rheostat, and each instrument is calibrated by a photometer to operate at a certain voltage; the reflecting surface is paper (English Cartridge Paper, 100 pounds), is 12 inches square, and covers a metal shield from which two rectangles  $\frac{3}{4}$  inch by  $\frac{3}{16}$  inch are cut out. (3) Behind

the metal shield is a dim light, so placed as to shine evenly through the two rectangles. In passing through the paper this light loses 90 per cent of its intensity and at the eye has an intensity of about 4 millifoot-candles, and is slightly reddish. A mechanical device controlled from the back of the box is used to cover one of the rectangles of dim light, while the other remains visible, the subject then having to distinguish between vertical and horizontal directions.

*Description of a Test.*—The subject adjusts his head comfortably against the rubber forehead rest, wearing glasses if they are usually worn, and is shown the rectangles of dim light, one "Up," one "Across." The bright light is put on, and he is asked to look at a black spot marking the position where the bar of dim light will later appear when the bright light goes out. During this period of thirty seconds, the name, date, etc., can be recorded on a card and the voltage checked. As the watch hand crosses thirty seconds the bright light is turned off and the subject reminded to say "Up" or "Across," indicating the direction of the rectangle seen, as soon as the direction can be made out. The time is noted when this is said, and represents the recovery time from thirty seconds of standard bright light.

Tests are repeated at minute intervals, three usually sufficing. In some cases the three values may be rather divergent, especially when the first value is long. It is then considered more accurate to use the median or midvalue, rather than the mean; e.g., of three values 23, 15, 16, use 16 rather than 18. The range of recovery times is from three to sixty seconds, with a mode at eight. Thirty to 40 persons an hour have been tested by one operator with one instrument.

*Discussion of Apparatus.*—Previous investigations in this field have been hampered by instruments too slow and complicated to permit the testing and retesting of large numbers of people, thereby lacking statistical weight for their findings. Hence the insistence on a test that would be rapid and simple. Every step in the development of this design was tested on numerous subjects not for the accuracy with which it would show new laws of optic physiology, but rather for the avoidance of complications in practice when testing for vitamin A deficiency. Repeated tests were made on each person at each stage, permitting calculations of means, and their standard errors, thus giving mathematical assurance of the validity of every modification. The result is a nice balance, obtained by experimentation, of numerous factors, including the color, duration, and intensity of the bright light, the size, color, duration, and intensity of the dim light, and the area and location of the retina affected and other points.

Reflection of the bright light from paper makes it more diffuse than reflection from or transmission through glass. Diffusion of the light helps to avoid the afterglow in the eyes from bright spots of light that causes some confusion otherwise. Confusion may also be lessened by having the outer edges of the reflecting surface actually, but not apparently, brighter than the center where the dim light is to be seen. This is done by having a circle of lights for bright illumination.

Having the dim light in the form of a small rectangle which may be vertical or horizontal gives a more constant recovery time and eliminates guessing which



is common in children. A suitable size was found that would be independent of astigmatism in the subject. Glasses are worn, if they are usually worn, only for the sake of speed, since no effect of removing them has been found, even in extreme myopia and hyperopia. There must, of course, be light transmission and perception. The terms "Up" and "Across" are used because they are much more familiar to the average subject and much faster than any others tried.

Since the standard error of the mean has been found to be about one second for many people, checks are required only within about one second, and the "reaction times" of subject and operator are unimportant. A practice effect—tests gradually getting shorter—has sometimes been observed if more than three tests were made at one-minute intervals, but if the interval was lengthened to five or ten minutes this effect disappeared.

The relatively large error permitted prevents significance being attached to various factors that might influence the test. The afterglow has already been mentioned, but it has too small an influence. The rubber forehead rest cuts out enough light so that the room need not be darkened, provided no light is shining from directly behind the subject. The fixation point (black spot) holds the light on the fovea centralis, but the area of retina covered is about  $3^\circ$ , so rods are involved as well as cones, and the results are satisfactory. Blue or gray eyes admit more light to the fundus than brown and might be more affected by the bright light, but with the accuracy demanded no relation to eye color has been found. Similarly, age, sex, and fatigue seem not to affect this test.

Other factors not related to vitamin A might show an effect, but in general they do not. The size of pupil would have an effect if the light reflex is defective. Experiments on several people with homatropine in the eyes showed a much prolonged recovery time (five to ten times normal) with widely dilated pupils. Measurement of pupil size, as it returned to normal, showed a linear correlation with the recovery time. Full contraction of the pupil with pilocarpine showed no effect. Marked differences between the two eyes and lack of coordination may influence the values, particularly by making great variations in successive tests. Experimentally covering one eye doubles or trebles the time taken when the two eyes were used, though commonly both eyes gave the same individual value.

## II. RELATION OF THE TEST TO VITAMIN A

*Distribution of "Recovery Times."* What Is "Normal"?—The frequency distribution of 1,519 people is shown in Fig. 2. The pronounced dip at eleven seconds suggests that this is a bimodal curve, which means a combination of two curves, each with its own mode. The two curves would be the distribution of (a) people "normal" in vitamin A and (b) people "deficient" in the vitamin, and are suggested by the broken lines. Assuming that the most common value (mode) of eight seconds is the mode of the distribution curve for normals, how can one decide the dividing line? An arbitrary standard has to be set up, just as all workers in this field have set up arbitrary points. In the beginning of

this work it was decided that any person with a "recovery time" of 11 or more seconds would be considered "deficient" in vitamin A, and that the deficiency would be greater as the time was longer. This arbitrary division is still used, though it errs in some cases, and receives support from studies of the response of vitamin A deficient people to vitamin A therapy.

Any person who does not show a change of recovery time after one dose of 50,000 I. U. of vitamin A, and who has no evidence of vitamin A deficiency is now considered "normal" in vitamin A. If a response is shown, it always consists of a shortened recovery time to a point where further treatment produces no effect. Such a person is considered "cured," in the absence of other signs of vitamin A deficiency. Hundreds of people have been so treated, and the work is being continued. Some have responded, some have not, and there is a surprisingly sharp division at the originally arbitrary time of eleven seconds.

*Seasonal Variation.*—A large group of students has been retested at intervals during the winter. About 500 students, equally divided into "normals" and "deficients" in their October tests, were involved. In the January retests of students who were normal in October, 26 per cent remained about the same value, 26 per cent had longer values (slightly deficient), while 48 per cent became definitely deficient (over 10 seconds). Of students who were deficient in vitamin A in October, 21 per cent became normal (mostly by treatment), 24 per cent remained about the same, and 55 per cent became more deficient. These figures show a clear tendency for adults to become more deficient as the winter goes on.

*Evidence of the Test's Relation to Vitamin A.*—The relation of this test to vitamin A is supported by the following evidence: (1) Of 1,500 tested, of whom 780 were "deficient" in vitamin A by the above standards, some 200 were given vitamin A. All, except 3, showed a reduction in recovery time. (2) The recovery time of a normal person was lengthened by taking a vitamin A-free diet, then reduced by vitamin A treatment. (3) Clinical observations on 4 patients with cirrhotic liver disease, 14 diabetic persons and 12 persons with gastric ulcers on Sippy diets, suggest a relation between dietary vitamin A or provitamins and the test. (4) Considerable subjective evidence of vitamin A deficiency with long recovery times and of improvement accompanying reduction of recovery time has been adduced.

1. *Response to Vitamin A by Deficient Cases.*—Two curves in Fig. 2 show the results obtained with the 200 people treated. All these people were retested during treatment until a constant recovery time was obtained, though usually this took only a short time. Repeated tests on controls, who received a vitamin A-free oil, showed no change from day to day. In Fig. 2 the curve with square dots shows the distribution of the untreated cases, from seven to forty-four seconds, and the round dots show the distribution after receiving vitamin A. The latter curve is sharply distributed around a mode of eight seconds, and is almost perfectly symmetrical between four and twelve seconds. These people are by definition "normal" in vitamin A, and the fact that their distribution curve is symmetrical about eight, supports the contention concerning a "normal" part of the large curve shown.

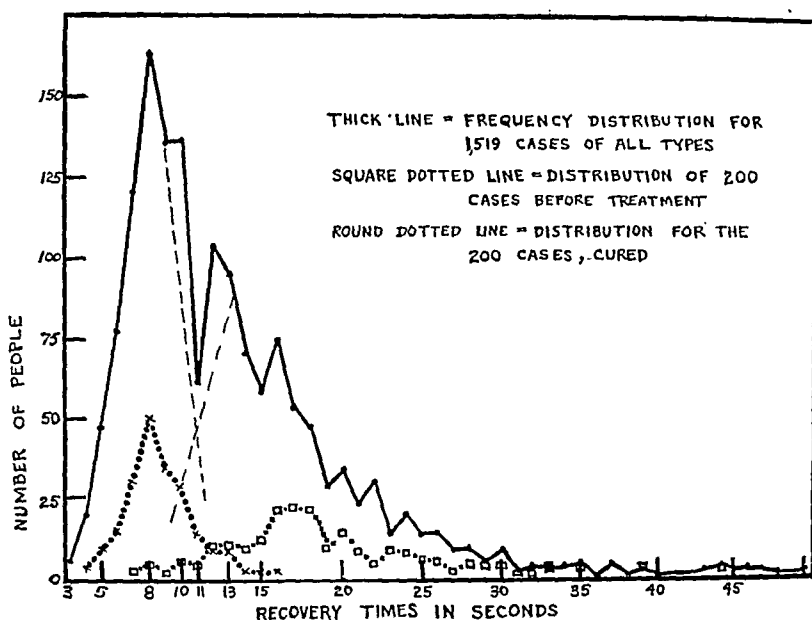


Fig. 2.—The number of people at each recovery time in this test. The dotted line shows the number of people cured by vitamin A at each time and is thus the distribution of normal values.

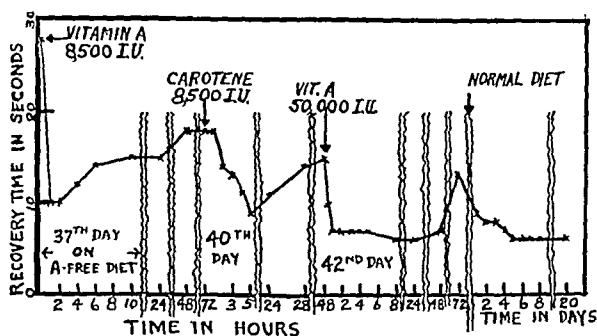


Fig. 3.—Time effects of small doses of vitamin A and carotene on the recovery times of a subject after thirty-seven days on a vitamin A-free diet. Increase in recovery time indicates the vitamin A deficiency.

*Treatment and Dosage.*—Treatment has been found most satisfactory when liver oil (usually halibut) of high vitamin A and low vitamin D content was used in capsules. Doses of 35,000 to 50,000 I. U. daily for three to five days usually resulted in a normal recovery time, and were much better than corresponding total dosages given 5 to 10,000 I. U. daily. People treated to this extent sometimes remained "normal" with no further supplement, but usually required about 5,000 I. U. daily for a further variable period of time, ascertained by tests. A number of people have been found who could only be maintained on doses of 10,000 I. U. daily or more. This is perhaps related to faulty absorption. In any case there has thus been established a wider individual variation and a higher daily requirement than has hitherto been described. Such people have invariably noted subjective improvement in various respects after the discovery of a high requirement and after subsequent treatment.

2. *Response of Normal Person to a Vitamin A Deficient Diet.*—An individual (myself) whose recovery time had been eight seconds for some months, and who had never experienced night blindness, eyestrain, dry skin, scanty saliva, or other signs of vitamin A deficiency, took a vitamin A-free diet for forty-five days. This diet was shown to contain less than 50 I. U. daily. The tests and coexistent symptoms are given in Table I for the first thirty-seven days, while Fig. 3 shows the effects of small doses of vitamin A or carotene for the remainder of the test period.

TABLE I

THE LENGTHENING OF THE RECOVERY TIMES OF INDIVIDUAL ON A VITAMIN A-FREE DIET,  
TOGETHER WITH ACCOMPANYING SYMPTOMS

DAYS ON VIT. A-FREE DIET	RECOVERY TIME SECONDS	COMMENTS
0	8	Same value daily for previous two weeks
1-9	8	
10-17	9	
18-22	11	Anorexia, which persisted two weeks
23-25	12	Mild diarrhea, persisted one week
26	14	Sore throat; rhinitis
27	15	
28, 29	16	"Cold" cleared up
30	17	Saliva noticeably decreased from now on
31, 32	18	Skin dry and scaling
33-35	20	Small gingival ulcers, night blindness
36	22	Eyes painful; no corneal ulcers
37	27	Epithelial casts in urine

The slow response shown in Table I, in which twenty-eight days were needed to reach a seriously deficient recovery time, may be explained on the basis of vitamin A and carotene storage in the liver. Moore<sup>18</sup> has shown that large reserves are carried even in a variety of serious diseases, and by calculation 1,000,000 I. U. may be a conservative estimate of the average person's reserve supply. It is not necessary to assume that this reserve must be exhausted before an effect be noted in a test, since some conservation may be expected. But work on experimental animals has shown that considerable depletion of liver carotene and vitamin A occurs before even the earliest metaplasia of epithelial tissues is observed (Bessey and Wolbach<sup>19</sup>).

As noted in Table I, signs of vitamin A deficiency were observed: anorexia, diarrhea, alalia, painful eyes, and photophobia, gingival ulcers, dry and scaling skin, nyctal-hemeralopia, urinary epithelial casts.

Fig. 3 shows that treatment with only 8,500 I. U. of vitamin A brought intense salivation and immediate response in the recovery times. These returned to the normal eight-second level for two hours, but gradually increased to deficient levels, presumably as the vitamin was removed from the blood. An equivalent dose of carotene (kindly supplied by the S. M. A. Corp.) brought a slower response, requiring five hours to attain the same level. This might be due to slower absorption from the gut, or to the need of conversion into vitamin A by the liver before affecting the test. Absorption in both cases was complete, as shown by analyses of urine and feces. A dose of

50,000 I. U. of vitamin A resulted in a recovery time of six seconds (lower than the previous base level). Shortly afterwards a normal diet was resumed, and after some days a recovery time of six seconds was attained.

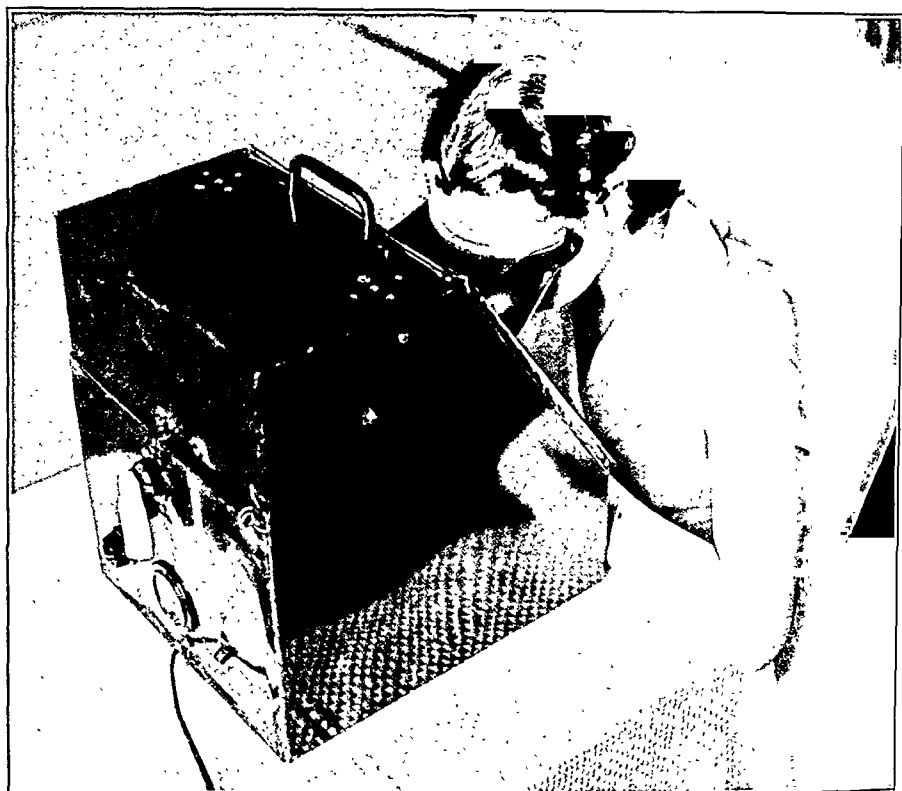


Fig. 4.

The disappearance of symptoms of vitamin A deficiency is of considerable interest. The salivation following intake of vitamin A on the thirty-seventh day of the vitamin A-free diet was over in twenty-four hours and marked the restoration of salivary flow, at first somewhat scarce. The painful eyes, which had been wrongly suspected of being due to corneal ulcers, were completely relieved on the thirty-ninth day. The gingival ulcers and night blindness seemed improved on the forty-second day, and were gone on the forty-fifth day. Sealing of the skin continued for about three weeks, and epithelial casts were found in the urine for five weeks.

Similar studies have been reported by Hecht and Mandelbaum,<sup>20</sup> and by Wald, Jeghers, and Arminio<sup>21</sup> but without any complete consideration of attendant symptoms.

3. *Clinical Evidence.*—(a) Liver disease. Haig, Hecht, and Patek<sup>22</sup> reported that 11 persons with cirrhotic liver disease had poor dark adaptation and were beneficially treated by large doses of vitamin A. These observations have been confirmed on 4 cases with values of twenty-two, twenty-seven, thirty-six, and fifty seconds. Presumably the conversion of carotenoids (provitamins) in the

livers of such people is impaired. Since the apparent vitamin A content of most diets is due to the carotenoids, impaired conversion leads to a deficiency even on an apparently adequate diet.

(b) Diabetic persons. Such a condition has also been found among diabetic persons where some liver dysfunction is usually present, and the diet contains large amounts of carotenoids. In 14 persons with diabetes, every case showed an apparent deficiency in vitamin A by this test, values ranging from thirteen to forty-five seconds. Further work is required on this new and interesting point.

(c) Gastric ulcers and special diets. A different type of vitamin A deficiency may be caused by special diets, restricting the intake of certain foods. Some of the most vitamin A-deficient cases encountered were found to be on Sippy or other ulcer diet, or on a diet for cholecystic disease. Values ranging from eleven to fifty-seven seconds were recorded. It seems not to have been realized that these diets are seriously deficient in vitamin A. In 12 ulcer cases specially treated with vitamin A concentrates more rapid improvement than expected was noted, and in one case a tendency to recurrence seems to have been checked. These observations must be extended before a full report can be made.

Such observations are in accord with the theory that lack of vitamin A may aggravate diseases in epithelial tissues (including mucous membranes) and may help their healing, without being directly responsible for the disease.

4. *Subjective Observations Related to Deficiency and Treatment.*—It is commonly claimed that these tests for dark adaptation, etc., provide the earliest clinical signs of a vitamin A deficiency, and it is tacitly assumed that a slight deficiency is a more or less serious condition which should be corrected. Few attempts have been made, however, to link these slight deficiencies with known clinical conditions. The most important clinical work has been done by Feldman,<sup>23</sup> but his study lacks statistical backing. The importance of poor vision at twilight (night blindness) in car accidents has recently been much emphasized.

A start on this problem of correlating symptoms with an objective test has been made by requiring students to answer certain questions at the time of being tested, and following up the answers later. A total of 753 such case histories have been summarized in Table II.

TABLE II

PERCENTAGE OF THE "NORMAL" GROUP (393 PERSONS) AND OF THE "DEFICIENT" GROUP (360 PERSONS) FOUND TO BE SUFFERING FROM VARIOUS CONDITIONS

CONDITION	PER CENT OF NORMALS	PER CENT OF DEFICIENTS
Consciously night blind	7	18
Eyestrain and photophobia	25	36
Dry prickly conjunctiva	5	10
Dry skin	7	17
Dry nasal mucosa	15	19
Scanty saliva (asialia)	2	6
Cold within a month of test	54	69
Average duration of colds	4 days	8 days

Many people found to be deficient in vitamin A were treated or treated themselves with various sources of vitamin A during several months. Most of these cases were retested, and often volunteered evidence of subjective improvement, even while unaware whether the test itself had shown improvement. This type of evidence is not capable of accurate expression, and involves so many psychic and other factors that it is commonly valueless. Nevertheless, some cases have been so striking as to command attention, and one such case will be described briefly.

A local airport attendant had to send up nightly a small meteorologic balloon with a dim light attached to it, and then follow its progress with a telescope. Sometimes he could not see the balloon for some time. On examination he was found to be "deficient" in vitamin A. Treatment brought forth obvious improvement in his ability to spot the balloon, as well as a shortened recovery time. It is likely that many around an airport are so affected by the beacons and ground lights that they cannot see dim lights quickly. There may even be greater demands on their system for vitamin A.

In any case it is clear that the test here described provides a rapid means for distinguishing people who are unadapted to some kinds of work, or who are working inefficiently, all depending on the recovery time from bright lights.

### III. PREVALENCE OF VITAMIN A DEFICIENCY

The original studies of Jeans and Zentmire<sup>24</sup> showed a vitamin A deficiency among children as high as 56 per cent. Similarly, Maitra and Harris<sup>25</sup> have reported studies on various groups of children with a deficiency averaging about 30 per cent for the 193 studied. Both these reports present a limited number of cases and attempt to draw conclusions on small groups of 20 and 30, owing to the inherent slowness and complication of the instruments used. Nevertheless, the chief arguments at the present time concern the probable extent of vitamin A deficiency, and the results on a large number of people are, therefore, especially important. Table III presents the results for different classes and ages of people so far studied. This type of work is now being extended.

TABLE III

PERCENTAGE OF PERSONS IN VARIOUS GROUPS FOUND TO BE DEFICIENT IN VITAMIN A  
BY THE TEST REPORTED IN THIS PAPER

GROUP	TOTAL NUMBER	PER CENT DEFICIENT
All cases to date	1603	52
Children (ages 8-15)	69	49
Freshmen (ages 17-20)	225	51
Freshettes	130	52
Other university students (ages 18-23)	640	52
Single men on relief (ages 20-35)	90	64
Other adults (over 25)	449	53

No special comment on the table is necessary except to note that the per cent deficiency in this district—52 per cent—seems a little higher than reported elsewhere. It can also be observed that age and sex made no significant difference to this figure, but that men on relief showed a higher percentage. The latter figure requires more extended observations before comment.

## SUMMARY

1. Details are given of a rapid visual test for vitamin A deficiency, depending on the recovery time after looking at a bright light. One test takes less than one minute, three tests being done within 5 minutes of the subject entering the room. This rapidity is valuable where large groups of people are concerned. The application to airmen, motorists, and the like, is pointed out.

2. Proof of the test's relation to vitamin A has been given by: (a) a report of experimentally induced vitamin A deficiency, correlating the test with other signs; (b) the prolonged recovery times observed in people with liver disease (cirrhosis or diabetes) or on special diets, whereby they either do not receive the vitamin or do not convert it from carotenoids; (c) the recovery on treatment with vitamin A of some 200 cases classed as deficient; and (d) subjective improvement in many such cases.

3. The effects on the test of sex, age, color of eyes, myopia, hyperopia, astigmatism, wearing of spectacles, and fatigue are negligible, since great accuracy is not demanded. The size of pupil, disparity of vision of the two eyes, and diseases might have an effect. These points are discussed.

4. Of 1,600 people examined 52 per cent have been found deficient in vitamin A, the incidence being higher among unemployed.

5. A table is given showing a higher incidence among vitamin A deficient people of night blindness, eyestrain, dry conjunctiva, dry skin, scanty saliva, and frequency and duration of colds.

6. Adults tended to become more deficient in vitamin A in February than they were in October.

It is unfortunately impossible to mention all the people who have helped in these investigations, either by patience in being tested, or by discussions. Special thanks for cooperation are due to M. R. Levey, M.D., C.M., D.L.O., of the Department of Ophthalmology; to D. E. Smith, Ph.D., of the Department of Psychology; to M. M. Cantor, B.S., M.D., to A. McKeever and to R. Clelland.

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## THE EFFECT OF HYPOTHALAMIC LESIONS ON FEVER INDUCED BY INTRAVENOUS INJECTION OF TYPHOID-PARATYPHOID VACCINE\*

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THE current conception of the nervous regulation of body temperature during the course of a disease like malaria is that, preceding the chill and period of rising temperature, the temperature-regulating mechanism suddenly becomes set at a higher level, with resultant increased heat production (shivering) and decreased heat elimination (vasoconstriction). The fall in temperature occurs when the regulating mechanism again becomes set at a lower level (DuBois<sup>1</sup>). It is only within the last few years that the location of the temperature-regulating mechanism within the hypothalamus has become sufficiently well known to make a satisfactory experimental approach to this subject possible, and the experiments which have been reported in the literature have not contributed much to its elucidation.

The reactions of animals with various lesions of the central nervous system to pyrogenic agents have been described by numerous investigators (among others, Freund and Strassmann;<sup>2</sup> Citron and Leschke;<sup>3</sup> Isenschmid;<sup>4</sup> Freund and Grafe;<sup>5</sup> Balcar, Sansum, and Woodyatt;<sup>6</sup> Girndt;<sup>7</sup> Solari;<sup>8</sup> Keller and Hare;<sup>9</sup> Thauer<sup>10</sup>). From many of these papers it is difficult to draw definite conclu-

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sions. Frequently acute animals were used, often in a state of shock; usually not more than two to ten days were allowed for recovery from operation. Protocols are often incomplete, and microscopic verification of the extent of the lesions is lacking. In most cases it is not apparent that adequate care was taken to stabilize the animal's temperature before exhibition of the pyrogenic agent. Dosages were usually varied from animal to animal.

Citron and Leschke,<sup>3</sup> using rabbits, made sections of the medial hypothalamus at the anterior border of the midbrain. Such animals did not react with fever to injections of *Trypanosoma nagana*, staphylococci, colloidal suspensions of paraffin, or even to beta-tetrahydronaphthylamine, but they were apparently in severe postoperative shock. Isenschmid<sup>4</sup> found that midbrain animals or animals with cervical cord transection responded with temperature rises to injection of sodium salicylate and to beta-tetrahydronaphthylamine after their body temperatures had been previously stabilized in an incubator.

Pinkston, Bard, and Rioch<sup>11</sup> injected typhoid-paratyphoid vaccine intravenously into a dog with chronic bilateral ablation of the neopallium which spared the hypothalamus. The result was an essentially normal fever curve.

Girndt<sup>7</sup> found that many rabbits from which he had removed the greater portion of the cortex bilaterally, responded to injection of *B. coli* vaccine with temperature falls instead of rises; but that if the cortex were removed on only one side dorsally, the temperature response to *B. coli* vaccine was normal. It would not be surprising if these extensive decortications had produced considerable hypothalamic damage. Unfortunately, there was no histologic verification of the extent of the lesions. In animals with a second type of operation (unilateral, circumscribed, dorsal decortication), he obtained a normal febrile response; then, without anesthesia, he pushed a blunt cannula into the medial hypothalamus through the free space dorsally and through it injected various drugs to test their central antipyretic effect. The mere introduction of the cannula and injection through it of normal saline solution produced an irreversible hypothermia in several animals previously inoculated with *B. coli* vaccine; in many others it resulted in milder temperature falls. These observations are of special interest because of the important part which the hypothalamus is known to play in temperature regulation.

In the present investigation an attempt has been made to determine the effect of hypothalamic lesions in cats on the reaction of these animals to the intravenous injection of typhoid-paratyphoid vaccine.

#### METHODS

Electrolytic lesions of the hypothalamus (or prechiasmatic region in several cases) were made with the Horsley-Clarke stereotaxic instrument in 21 cats. The exact location of each lesion was determined by serial sections through the hypothalamus stained alternately by Weil's method and by cresyl-violet. The ability of each cat to regulate its body temperature was studied in considerable detail both preoperatively and postoperatively by means of numerous tests in both hot and cold boxes.

Sixteen normal cats were subjected to intravenous injection of typhoid-paratyphoid vaccine to establish the normal response. Each of the 21 operated cats, after a convalescence of a month or more, was subjected to exactly the same procedure.

Each cat was brought to the laboratory about 8 A.M. and placed on a specially designed canvas animal board to which it was strapped comfortably but securely. The body temperature was recorded as a continuous curve by the ink-writer of a Leeds and Northrup resistance temperature recorder, the resistance unit of which was inserted through the rectum to a constant measured distance of 14 cm. from the anal orifice. An area 3 to 4 cm. in diameter on the side of the chest had been depilated the previous day, and to this area a skin thermocouple was applied by a spring-mounted holder mounted on a ring stand. The room temperature and humidity were read at frequent intervals from a wet and dry bulb thermometer ventilated at about 3 meters per second by an electric fan.\* In each case all the apparatus was mounted and set in function as soon as the cat was brought to the laboratory, and the resistance recorder traced a curve of the body temperature until this had become completely stabilized (often several hours). All temperatures are recorded in degrees Fahrenheit. After the temperature stabilization was accomplished, 0.35 c.c. per kg. of typhoid-paratyphoid vaccine was injected into the saphenous vein. This vaccine was all poured at one time from one freshly prepared stock bottle, kindly furnished by the Abbott Laboratories, Inc. It contained 1,000 million killed typhoid organisms, 750 million killed paratyphoid alpha, and 750 million killed paratyphoid beta organisms in each cubic centimeter. The dosage was not varied at any time. About 5 P.M. the skin thermocouple was dismantled and the fan ventilating the wet bulb thermometer was stopped. The setup was left otherwise undisturbed, tracing a constant record of the animal's temperature until the next morning, about twenty hours after injection.

In order to determine whether lesions in other parts of the brain would have a similar effect on the reaction of cats to typhoid-paratyphoid vaccine another series of experiments was made later but with a different lot of vaccine. This second lot was as nearly as possible of the same potency as the first, but it is not possible to be certain that the two series of experiments are altogether comparable.

#### THE NORMAL RESPONSE

The normal cat responds to intravenous injection of typhoid-paratyphoid vaccine with a cyclic response in which first the heat-preservation mechanism, then the heat-loss mechanism predominates. The periods of dominance of the heat preservation and elaboration mechanism are accompanied by shivering, slowing of the respiratory rate, and signs of generalized sympathetic stimulation, such as pupillary dilatation, cutaneous vasoconstriction, and erection of hair. The periods during which the heat-loss mechanism predominates are accompanied by marked acceleration in respiratory rate, by cessation of shivering, and by signs of generalized parasympathetic stimulation, such as pupillary constrict-

\*The maximal room temperature variation during the course of any one experiment was 2° F.; the maximal variation in relative humidity during any one experiment was 2 per cent.

tion, defecatory movements, urination, penile erection, and cutaneous vasodilatation; during such periods the temperature falls.

Usually immediately, almost always within ten to thirty minutes after injection, the temperature begins to rise, accompanied by shivering and signs of generalized sympathetic activity; it reaches a first temperature peak averaging  $1.2^{\circ}$  F. above the stabilized base line at an average interval of forty-eight minutes after the injection (Fig. 1). Shivering then ceases, respiration accelerates sharply, and under signs of marked generalized parasympathetic stimulation the body temperature drops sharply to a first intermediate low point which averages

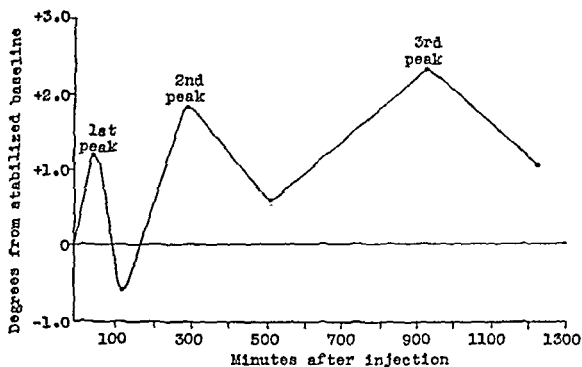


Fig. 1.—Composite fever curve, 16 normal cats, after injection of typhoid-paratyphoid vaccine intravenously

$0.6^{\circ}$  F. below the base line, reached on an average about two hours after injection. Now the animal begins to shiver, sympathetic activity again becomes predominant, and the temperature rises to a second peak, averaging  $+1.8^{\circ}$  F., reached on an average four and one-half hours after injection. Again the temperature falls, reaching another low point, averaging  $+0.57^{\circ}$  F., at an average eight and one-half hours after the injection, rising then to a third peak which averages  $+2.3^{\circ}$  F., fifteen and one-half hours after the injection. The temperature falls away slowly from this third peak but it is still well above the base line the next morning, averaging  $+1.06^{\circ}$ , twenty hours after the injection.

The extreme range of variation in the normal was represented by the normal cat of Experiment 22, which, after holding its temperature practically constant during the first forty-five minutes after injection, showed a rapid drop in body temperature thereafter, the temperature reaching a low point of  $2.3^{\circ}$  F. below the base line, three and one-half hours after the injection. From this point, however, the temperature rose, reaching  $0.4^{\circ}$  F. above the base line eighteen hours and forty minutes after the injection; at the termination of the experiment twenty-one hours after the injection the body temperature was exactly at the base line. The next most extreme variation from the average in the normal series of cats was shown by the normal cat of Experiment 26. During the first forty minutes this cat's temperature remained practically stationary, falling

only slightly. Then the temperature fell markedly, reaching a low point of  $3.5^{\circ}$  below the base line, one hour and forty-three minutes after the injection. From this low point, however, the temperature rose rapidly, reaching a high point of  $2.8^{\circ}$  F. above the base line, sixteen hours after the injection. The next morning, nineteen hours after the injection, the cat's temperature was still  $1.2^{\circ}$  F. above the base line.

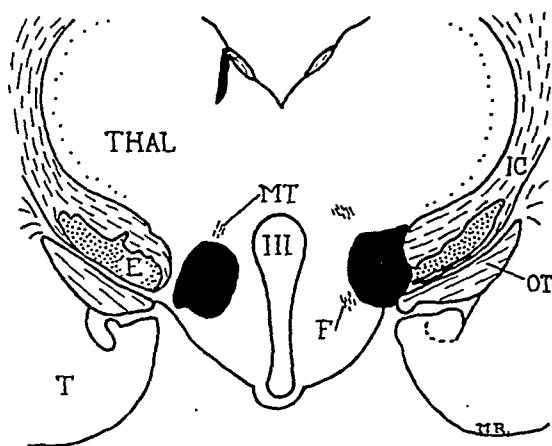


Fig. 2.—A diagrammatic drawing of a frontal section through the diencephalon of Cat 20 with bilaterally symmetrical lesions laterally placed in the rostral part of the hypothalamus. F, fornix; IC, internal capsule; MT, mammillothalamic tract; OT, optic tract; T, temporal lobe; THAL, thalamus; III, third ventricle.

#### RESPONSE OF CATS WITH HYPOTHALAMIC LESIONS

Cats with certain hypothalamic lesions, on the other hand, show strikingly different temperature curves after intravenous injection of typhoid-paratyphoid vaccine. The temperature falls many degrees below the stabilized base line, and the hypothermia persists so that the next morning at the termination of the experiment the temperature is still depressed  $4^{\circ}$  F. or more.

In this series of 21 cats with lesions of the hypothalamus (or in a few cases of the prechiasmatic region) there was not a single case which showed a deviation of the febrile response in the direction of an increase in the height or steepness of slope of the fever curve. Only one animal showed a typical normal fever curve. Six cats reacted to the vaccine with fever curves, which while falling within the range of the more atypical normal reactions to the vaccine, showed a definite deviation in the direction of smaller than usual rises and greater than average falls. Two animals responded to the injection of the vaccine with moderate falls in temperature. The rest of the series, 12 cats in all, showed marked and prolonged temperature falls which were well beyond any deviations from the average encountered in our normal series of cats.

Cat 20 will serve as an example. Bilateral lesions were placed in the lateral hypothalamus, as illustrated in Fig. 2. There resulted a slight impairment of ability to regulate against cold and a marked and prolonged loss of ability to regulate against heat. As late as sixty-seven days after the operation the cat failed to pant or sweat when it was placed in a hot box, although its rectal temperature was raised to  $106^{\circ}$ . It was injected with typhoid-paratyphoid vaccine

fifty-six days after the operation, and the marked and prolonged drop in temperature shown in Fig. 3 occurred. Seven days after the injection of the vaccine we injected into the same cat 20 mg. per kg. of alpha 1-2-4-dinitrophenol intravenously (3 per cent solution in sterile water containing 1.5 per cent sodium bicarbonate), the technique of the experiment being otherwise the same as when vaccine was injected. The temperature rose  $3.4^{\circ}$  in three and one-half hours and remained steady at about that level until the termination of the experiment, 5 hours after the injection, at which time the animal's temperature was  $3.3^{\circ}$  above the base line. Whatever it may have been that was responsible for the fall in temperature following the injection of the vaccine did not prevent dinitrophenol from producing fever.

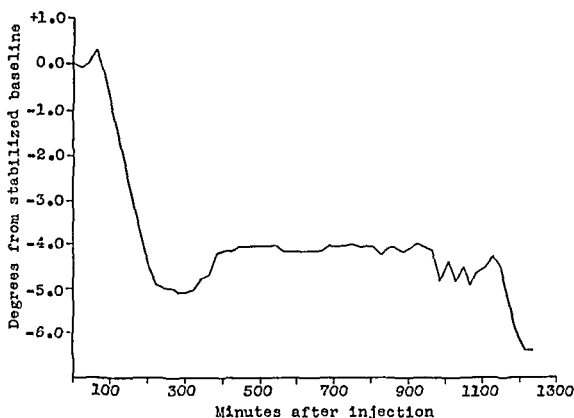


Fig. 3.—Chart showing marked fall of temperature in Cat 20 following intravenous injection of typhoid-paratyphoid vaccine.

In the period of the first fall of temperature after injection of the vaccine (fall from the first peak), the operated animals reacting with hypothermia showed an average rise in skin temperature due to vasodilatation of  $1.5^{\circ}$ , whereas those animals reacting with essentially normal curves showed an average rise due to vasodilatation of  $1^{\circ}$ . The corresponding figures for our normal, unoperated cats was  $0.4^{\circ}$ .

In the period of the first fall (fall from the first temperature peak), the average maximum respiratory rate attained by the cats reacting with hypothermia was higher than that attained by the animals responding with approximately normal curves (2.3 times the stabilized preinjection respiratory rate against 1.7 times; an absolute increase in respiratory rate of 50.3 per minute against 28 per minute). Shivering was about as often seen in those animals that reacted to the injection with hypothermia as in those that reacted with approximately normal fever curves. Eight out of 12 of the animals, reacting with hypothermia, shivered during the course of the experiment; 4 out of 6 of

the animals, reacting with fairly normal curves, shivered during the corresponding period of time. In a series of normal unoperated cats, 14 out of 16 shivered during the same period.

In those animals which responded to the injection of the vaccine with hypothermia, there was very often during the first period of thirty to forty minutes pupillary constriction, defecation, and salivation; occasionally the pupils were of medium size, seldom dilated. Something contained in the vaccine, therefore, stimulated the heat-loss mechanism, causing not only acceleration of respiration but also vasodilatation along with signs of parasympathetic activation.

In the second series marked falls in temperature resulted from injection of typhoid-paratyphoid vaccine in 1 out of 4 cats from which the frontal lobes had been removed and in 2 out of 10 cats with lesions in the dorsal thalamus. Similar reaction to the vaccine was obtained from an undernourished but otherwise normal cat. This cat had been kept on an inadequate diet, a small quantity of milk with plenty of water, for seventy-six days until it had lost 49.8 per cent of its original body weight and had then been given a full diet for seventeen days and at the time of injection had reached 72 per cent of its original weight. Two unoperated cats which were sick with distemper also gave drops instead of rises in temperature as a result of vaccine injection. These observations tend to throw doubt on the specificity of the reactions obtained from the cats with hypothalamic lesions. It is possible that several factors may be responsible for these abnormal reactions. But the fact that in the first series of experiments 12 out of 21 cats with hypothalamic lesions showed marked and prolonged temperature falls which were well beyond any deviations from the average encountered in the normal series, cannot be too lightly thrust aside. While a few of the 12 operated cats which showed marked drops in temperature were poorly nourished, the majority of them were in excellent condition, well nourished, and free from any obvious infection.

While there is a pronounced difference between the results obtained from cats with hypothalamic lesions and from those of the normal series, this difference must be interpreted cautiously. The drops in temperature in the cats with hypothalamic lesions did not appear to be dependent on damage to the hypothalamic heat-regulating centers and occurred in some cats in which these lesions had caused little or no disturbance in temperature regulation. It did not appear to be important whether these lesions were medially or laterally, rostrally or caudally placed. It is true that in the 6 cats with lesions in the caudolateral part of the hypothalamus, which showed markedly impaired ability to regulate against cold, 5 showed marked falls in temperature following the injection of typhoid-paratyphoid vaccine and only one showed a normal fever curve. This one cat and 2 others, i.e., 3 of the 6, belonged to the second series of experiments. But just as marked falls were obtained in some other cats in which there was no obvious disturbance in temperature regulation.

#### DISCUSSION

In a paper dealing with the thermal reactions induced by the intravenous injection of typhoid-paratyphoid vaccine in normal cats (Ranson, Jr.<sup>12</sup>), evi-

dence was presented which seems to show that the vaccine stimulates both the mechanism for conservation of bodily heat, including the sympathetic system, and the mechanism for heat loss, including the parasympathetic system. There is a great deal of evidence that the hypothalamus serves as a general sympathetic center and that its destruction interferes with heat production and conservation. The present investigation was begun with the idea of studying the effect on febrile reactions of the destruction of this center. It soon became obvious, however, that typhoid-paratyphoid vaccine which had been chosen as the fever-producing agent does not always cause a typical fever in normal cats and that its action is influenced by a number of obscure and uncontrollable factors. Because of this difficulty the investigation has not yielded as much information as might be desired in regard to the role of the hypothalamus in fever. Whether it does or does not play a part remains undetermined. The frequent occurrence of marked drops in temperature instead of rises in cats with hypothalamic lesions might be interpreted in favor of such a role, but, on the other hand, the absence of any correlation between the amount of disturbance in temperature regulation caused by the lesions and the character of the temperature curve following typhoid-paratyphoid injection may be taken as evidence to the contrary. Furthermore, in one cat of the second series, in which the lesions were placed bilaterally in the caudolateral part of the hypothalamus and produced a marked impairment in ability to regulate both against heat and cold, the vaccine caused a fairly normal fever reaction with a rise in temperature of  $3^{\circ}$ . If this experiment may be taken at its face value it would seem to indicate that the vaccine may cause fever by stimulating the heat-production and heat-conservation mechanism at a point below the hypothalamus.

One point, however, is clear. The marked falls in temperature, which occur in some cats following injection of typhoid-paratyphoid vaccine, are not due to the stimulation of that part of the heat-loss mechanism which lies in the hypothalamus and preoptic region. It has been shown that the receptive part of the mechanism lies in the preoptic region, that it is activated by moderate rises in temperature, and that a pathway leads backward from it through the lateral part of the hypothalamus to a heat-loss mechanism located behind the hypothalamus. When this descending pathway from the anteriorly placed heat-sensitive center is interrupted by bilaterally symmetrical lesions in the lateral part of the hypothalamus, the animal loses its ability to protect itself against overheating (Magoun, Harrison, Brobeck and Ranson,<sup>13</sup> Clark, Magoun and Ranson<sup>14</sup>). In 4 cats of the first series and one of the second, in which the lesions had been so placed as to interrupt this descending path bilaterally and to prevent the animals from panting or sweating when the rectal temperature was raised to  $106^{\circ}$ , injection of typhoid-paratyphoid vaccine caused marked drops in temperature. This shows that the part of the heat-loss mechanism which is stimulated by the injection lies caudal to the hypothalamus.

#### SUMMARY

Of 21 cats with chronic lesions of the hypothalamus (and in a few cases, prechiasmatic region) there was not a single case which showed a deviation of the fever curve in response to the intravenous injection of typhoid-paratyphoid



vaccine in the direction of an increase in height or steepness of slope. Only one animal showed a typical normal fever curve. Twelve showed marked and prolonged temperature falls ( $3^{\circ}$  to  $11^{\circ}$  F.) which lasted for a period of twenty or more hours.

Although such animals shivered normally in response to the injection of the vaccine, the temperature did not rise appreciably in response to such shivering; the pupillary dilatation which usually accompanies such periods of shivering and rising temperature in the normal animal was more often absent in these hypothalamic animals responding to the vaccine with hypothermia. Instead, there was pupillary constriction or the pupil was medium. There were often coincident signs of parasympathetic overactivity, such as defecation, urination, and salivation. The respiratory rate increased markedly, greatly exceeding that seen in the normal series of cats.

It is impossible at this time to make any definite anatomical localization for lesions resulting in this reaction. It does not appear to be important whether the lesions are laterally or medially, rostrally or caudally placed, or whether the lesion results in a disturbance in the normal regulation of body temperature.

A hypothalamic lesion which results in a hypothermic reaction to the injection of typhoid-paratyphoid vaccine intravenously does not prevent the animal from responding to the intravenous injection of alpha-dinitrophenol with a typical febrile response.

The part of the heat-loss mechanism which is activated by the intravenous injection of typhoid-paratyphoid vaccine does not lie in the hypothalamus but somewhere caudal to it.

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## THE RELATIONSHIP OF THE INSULIN HYPOGLYCEMIC REACTION TO SHOCK<sup>\*</sup>

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THE term *shock* is commonly applied loosely to various conditions of disease, quite diverse in character. Moon has segregated one definite entity from the heterogeneous group by the presence of a characteristic combination of physiologic features. He defined shock as a circulatory deficiency not cardiac and not vasomotor in origin, characterized by decreased blood volume, decreased volume flow, and hemoconcentration. He showed also that this syndrome is accompanied by characteristic pathologic changes seen post mortem. These include widespread capillovenous congestion and evidences of increased capillary permeability such as edema, effusions, and petechiae in visceral areas.

Hypoglycemic manifestations, resulting from the administration of insulin, are often spoken of as "insulin shock." It seems pertinent to determine whether this reaction has the characteristic features of shock as defined. This can be done by determining the presence or absence of hemoconcentration during "insulin shock" and by noting changes present in the viscera after death from large doses of insulin. Experiments on dogs and rabbits were arranged to secure data on these points.

The dogs employed were of both sexes and ranged from 8 to 15 kg. in weight. There were 4 white rabbits and 2 rabbits of the Dutch breed. These were all young adult males, apparently in perfect health and weighing approximately 2 kg. each. The variability in their insulin tolerance perhaps was related to a factor of sex. Dotti has reported that bucks are much less constant than does in their responses to insulin injections. All animals were maintained on standard kennel rations. A fasting period of eighteen hours preceded each of the experiments on dogs. The rabbits were not fasted, a fact which may have further added to the variability in their tolerance to insulin. Hemoglobin determinations, red blood cell counts, and determinations of the erythrocyte volume were made in all instances on venous blood. The blood was obtained from the jugular vein in the dog and from the marginal vein of the ear in the rabbit. In dogs the hemoglobin determinations were made by the Sheard-Sanford photometer;<sup>16</sup> in rabbits the Haden-Hausser clinical hemoglobinometer was used. Van Allen hematocrit tubes were employed in determining the volume of erythrocytes.

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The dogs were divided into three groups:

*Group 1:* Three dogs received intravenous injections of 1 unit of insulin per kg. of body weight every two hours until convulsions occurred. They were then resuscitated by intravenous injections of glucose. The blood was examined at hourly intervals (Table I).

TABLE I

DOGS RECEIVING 1 UNIT OF INSULIN PER KILOGRAM OF BODY WEIGHT EVERY TWO HOURS UNTIL CONVULSIONS OCCURRED

TIME IN HOURS	0	1	2	3	4	5	6	7	8	9	10
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*Dog 1*

R.B.C. in millions	8.05	8.05	8.50	7.75	8.00	7.50	7.10	8.50	8.25	7.90	8.30
Hemoglobin in grams	19.2	20.8	19.0	16.8	17.2	16.0	15.2	17.4	18.1	19.0	17.8
Hematocrit reading	52	56	54	52	50	46	50	46	51	49	48
W.B.C. in thousands	11.5	10.0	12.1	16.9	15.8	14.4	17.4	19.9	16.8	19.0	18.8

*Dog 2*

R.B.C. in millions	7.80	8.05	8.20	8.20	7.95	8.10	7.80	8.70			
Hemoglobin in grams	16.4	18.6	18.0	16.6	18.2	17.4	16.8	18.8			
Hematocrit reading	50	54	54	51	53	49	53	56			
W.B.C. in thousands	10.1	8.70	10.4	12.3	14.0	14.2	14.8	15.7			

*Dog 3*

R.B.C. in millions	7.91	8.50	8.10	7.95	7.55	7.13	7.05	8.35			
Hemoglobin in grams	17.2	17.8	17.6	16.8	16.6	17.2	15.2	15.8			
Hematocrit reading	51	55	53	52	48	51	47	48			
W.B.C. in thousands	10.8	13.2	17.3	23.3	18.6	20.2	16.4	16.2			

TABLE II

DOGS RECEIVING 2 UNITS OF INSULIN PER KILOGRAM OF BODY WEIGHT

(Convulsions occurred early because these animals had been subjected to insulin experiments on the previous day. See text.)

TIME IN HOURS	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$
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*Dog 4*

R.B.C. in millions	8.40	7.90	8.00	7.75	8.30	8.05
Hemoglobin in grams	18.0	17.6	18.2	17.5	18.0	17.6
Hematocrit reading	53.5	54.0	52.0	49.5	54.0	53.0

*Dog 5*

R.B.C. in millions	6.87	6.90	6.65			
Hemoglobin in grams	14.2	14.6	13.4			
Hematocrit reading	49.0	49.0	50.0			

*Dog 6*

R.B.C. in millions	7.04	7.20	6.80	7.05		
Hemoglobin in grams	17.4	17.0	14.8	16.6		
Hematocrit reading	55.0	53.0	52.0	53.5		

TABLE III

TWENTY UNITS OF INSULIN PER KILOGRAM OF BODY WEIGHT ADMINISTERED TO DOG 7; 25 PER KG. TO DOG 8

("C" indicates the time at which the first convulsion occurred; "D," the time at which death ensued.)

TIME IN HOURS	0	4	6	6½	7	7½	8½	8¾	9	12	22	25
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*Dog 7*

R.B.C. in millions	7.15	7.50	C	7.00					7.65	7.20	7.30	D
Hemoglobin in grams	13.0	12.8	C	14.2					14.4	13.4	14.2	D
Hematocrit reading	44	48	C	43					46	44	46	D

*Dog 8*

R.B.C. in millions	6.45	6.60			C	6.85	6.50	D				
Hemoglobin in grams	14.2	13.0			C	14.0	14.5	D				
Hematocrit reading	42	40			C	44	42	D				

TABLE IV

RABBIT 1 RECEIVED 20 UNITS OF INSULIN PER KILOGRAM OF BODY WEIGHT; RABBIT 2, 30 UNITS PER KG.; RABBIT 3, 75 UNITS PER KG.; AND RABBIT 4, 100 UNITS PER KG.

TIME IN HOURS	0	2	2½	3½	4½	5	6	6½	6¾	8	9½	9¾
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*Rabbit 1*

R.B.C. in millions	5.85	C	5.75	5.50	D							
Hemoglobin in grams	11	C	11	11	D							
Hematocrit reading	32.5	C	34.5	33.5	D							

*Rabbit 2*

R.B.C. in millions	5.85					C		6.10	6.05	5.90	D	
Hemoglobin in grams	11					C		12	12	11	D	
Hematocrit reading	34					C		33.5	33	34	D	

*Rabbit 3*

R.B.C. in millions	4.85							C	5.30	5.00		D
Hemoglobin in grams	10							C	11	10		D
Hematocrit reading	30.5							C	30.0	30.0		D

*Rabbit 4*

R.B.C. in millions	5.90			C	5.85	6.55		6.05	D			
Hemoglobin in grams	11			C	11.5	12.0		12.0	D			
Hematocrit reading	33.5			C	38.0	35.5		38.0	D			

*Group 2:* The three dogs in this group received 2 units of insulin per kg. of body weight intravenously (Table II). Convulsions occurred early in each of these dogs because on the previous day they had been subjected to repeated

small injections of insulin until convulsions occurred. Decrease in resistance to hypoglycemia on successive days of insulin administration has already been reported.<sup>3</sup> Blood samples were obtained on the animals of group 2 at half hourly intervals. Following the occurrence of convulsions, they too were resuscitated by injections of glucose.

*Group 3:* Table III summarizes the protocols of the two dogs of group 3. Large doses of insulin (20 to 25 units per kg., respectively) were given to these dogs subcutaneously. Several samples of blood were obtained after the onset of convulsions, which occurred at frequent intervals until death ensued. Post-mortem examinations were made on both of these animals. The viscera appeared normal in all respects. There were no evidences of dilatation and engorgement of the capillaries and venules. Neither petechial hemorrhages, edema, nor serous effusions were present in any of the viscera.

Four rabbits received single massive doses of insulin subcutaneously, and the concentration of their blood was determined at irregular intervals until death ensued (Table IV). Two rabbits were given nonfatal doses of insulin intravenously followed by larger doses twenty-four hours later. Similar blood studies were made on these animals (Table V). Post-mortem examinations were made on all of the rabbits immediately following death. Again there were no visceral evidences of capillovenous congestion or increased capillary permeability.

TABLE V

ANIMALS RECEIVING 10 UNITS OF INSULIN PER KILOGRAM OF BODY WEIGHT AT THE BEGINNING OF THE EXPERIMENT AND 20 UNITS PER KG. AT THE TWENTY-FOURTH HOUR

TIME IN HOURS	0	6	24	26	27½	28	28½	28¾	29¼
<i>Rabbit 5</i>									
R.B.C. in millions	6.35	6.60	6.85	6.50	C	6.80	D		
Hemoglobin in grams	12	12	13	12	C	13	D		
Hematocrit reading	37.5	38.0	36.0	37.5	C	39.0	D		
<i>Rabbit 6</i>									
R.B.C. in millions	5.25	5.05	5.20	5.55		5.65	C	5.30	D
Hemoglobin in grams	10	10	11	10		11	C	10	D
Hematocrit reading	31.5	32.0	30.0	32.5		33.0	C	31.5	D

The data assembled in the accompanying tables indicate that in these experiments hemoconcentration did not result from the intravenous or subcutaneous injection of nonfatal or fatal doses of insulin. Post-mortem examination revealed none of the visceral changes found in animals after death by experimental shock.<sup>13</sup>

## COMMENTS

Some data concerning hemoconcentration have been recorded in reports dealing with the effects of insulin. Drabkin and Edwards reported that large doses of insulin given to dogs were followed by anhydremia as indicated by an increased hemoglobin percentage. Levine and Kolars reported increased numbers of leucocytes and erythrocytes after injections of insulin in rabbits. They interpreted this increase as evidence of anhydremia. They did not state the dosage of insulin employed, but believed there was no relationship between the

size of the dose and the degree of anhydremia. Chaikelis made determinations of erythrocyte counts, the hemoglobin, and the water content of the blood of rabbits after small doses of insulin. He stated that a moderate anhydremia accompanied the hypoglycemia and confirmed the observation (Levine and Kolars) that the degree of anhydremia was unrelated to the dosage of insulin. Anhydremia during insulin hypoglycemia has been accepted to such an extent that many authors have incorporated it in theoretical explanations of various features of the insulin reaction.

Other reports are at variance with those cited. Mazzocco and Morera did not find increased numbers of erythrocytes during insulin hypoglycemia in dogs. Olmstead and Taylor found no significant change in the hemoglobin percentage after moderate doses of insulin in rabbits. In the dog an increase in the hemoglobin and the number and volume of erythrocytes may occur as the result of contraction of the spleen with subsequent emptying of its contents.<sup>7, 8, 9, 11</sup> However, in the rabbit<sup>9</sup> and in man, animals in which the spleen is comparatively smaller, less muscular, and much less active, hemoconcentration is almost entirely dependent upon loss of fluid from the vascular bed. Obviously this entails a decrease in the blood volume. Yet Kay and Smith found no changes in the blood volume of rabbits after the administration of insulin. Furthermore, Chaikelis concluded that the apparent decrease in the water content of the blood, which he reported in rabbits, could be shown to exist only by the statistical treatment of a large group of observations. He stated that the individual results were too varied and the degree of change was too small to be demonstrated by individual observations or even by short series of observations.

The data supplied by Drabkin and Edwards indicate that they actually were dealing with hemoconcentration. The conditions of their experiments were such that true shock may have originated from the combination of large doses of insulin, long anesthesia (injected intraperitoneally), and operative procedures. Indeed, in the one dog on which additional data were obtained, reductions of 53.1 per cent in the plasma volume, and of 42.1 per cent in the total blood volume were found. These findings indicate that shock, rather than hypoglycemia, was the major physiologic disturbance in these animals.

The increase in the number of leucocytes noted in Table I is in agreement with the findings of Levine and Kolars, Nitzescu and Mangiuca and others. Various explanations have been suggested for the occurrence of leucocytosis during insulin hypoglycemia. It probably results from the adrenalinemia consequent to the injection of insulin. That adrenalin causes lymphocytosis and neutrophilia, has been demonstrated by other investigators (Garrey and Bryan).

The absence of hemoconcentration and of post-mortem evidences of capillo-venous congestion and increased capillary permeability indicate that the mechanism of death from large doses of insulin is not related to the mechanism of shock. Further evidence of the dissimilarity of the two mechanisms is the prompt recovery from the insulin reaction obtained by the administration of sugar. There are no available means by which such a dramatic recovery may be obtained in shock.

## SUMMARY

1. Determinations of the hemoglobin content, and the number and volume of erythrocytes in the blood of dogs and rabbits, indicate that hemoconcentration does not occur incident to hypoglycemic reactions resulting from injections of nonfatal or fatal doses of insulin.

2. Visceral evidences of capillovenous congestion and increased capillary permeability were not seen in animals after death by insulin hypoglycemia.

3. The mechanism of death resulting from large doses of insulin is not the same as that of shock. The term "insulin shock" is confusing and should be abandoned.

4. The leucocytosis recorded in previous reports on insulin hypoglycemia was confirmed.

Since this article was written studies on the protein content and the colloid osmotic pressure of the serum in man and the dog during insulin hypoglycemia have been published by Butt and Keys (*Arch. Int. Med.* 63: 156, 1939). They concluded that "insulin shock" bears no close relation to other types of shock."

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# LABORATORY METHODS

## ADDITIONAL RECORDINGS OBTAINED WITH THE OSCILLATO-CAPACIGRAPH\*

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RECENTLY Fenning<sup>1</sup> described an instrument capable of recording, simply and graphically, small changes in capacity. Previous publications by Bonar and Fenning<sup>2</sup> included records of respiratory movements in small animals, uterine contractions in rats and guinea pigs, fetal movements and fetal respiratory movements. Since these studies were made, additional recordings have been obtained which show other possible physiologic and clinical uses of the instrument.

As previously implied, the instrument may be used to detect and record on photosensitive paper any activity which directly or indirectly produces a displacement of the surface of the body under investigation. Provisions must be made for placement of the plate used in conjunction with the instrument in proximity to, but not in contact with, the surface under investigation.

With proper adjustment of the circuit of the instrument, extremely high sensitivity is available. Fig. 1 well illustrates this fact. This figure represents the apex beat displacement recorded from the anterior thoracic wall of a rat fetus weighing 0.85 gm. The actual displacement in this case was difficult to see with the unaided eye. Reflected light from the moist, glistening surface of the thorax provided the necessary amplification factor for recognition of thoracic movement. Opening of the thorax disclosed a beating heart having a frequency identical to the observed and recorded displacements.

Fig. 2 is a recording obtained from a larger rat fetus with the thorax opened and the heart exposed. In this recording distinct auricular and ventricular components are recognized. An additional feature of this recording is the presence of a partial heart block having a variable rhythm—three and four to one. During the periods of ventricular inactivity the influx of blood produces an enlargement of the heart with a corresponding shift of the surface toward the plate. This change is recorded as a slower upward deflection, upon which are superimposed the deflections due to auricular activity. The slow rise terminates with an abrupt upward deflection, which is synchronous with ventricular activity. This is followed by an abrupt fall which is concomitant with the onset of ventricular inactivity. As the result of ventricular activity there occurs the ejection of blood and a corresponding decrease in cardiac size.

\*From the University of Utah Medical School, Salt Lake City.

†Due to the untimely death of Dr. Bonar, this report represents an attempt on the part of the author to call to the attention of investigators, work that was done in collaboration.

Read in part by Dr. Bonar at the Fourth Region Meeting of the American Academy of Pediatrics in Los Angeles, Calif., November 6, 1937.

Received for publication, December 16, 1938.



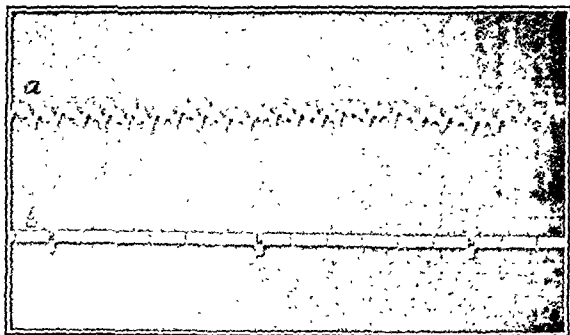


Fig. 1.

Fig. 1.—*a*, Cardiac beat. *b*, Time, one second.

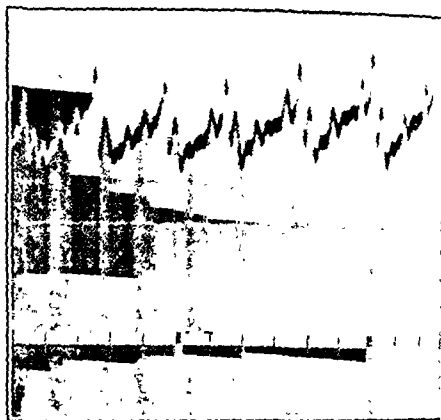


Fig. 2.

Fig. 2.—1. Cardiac activity. *a*, Auricular activity. *b*, Ventricular activity. 2. Time, one second.

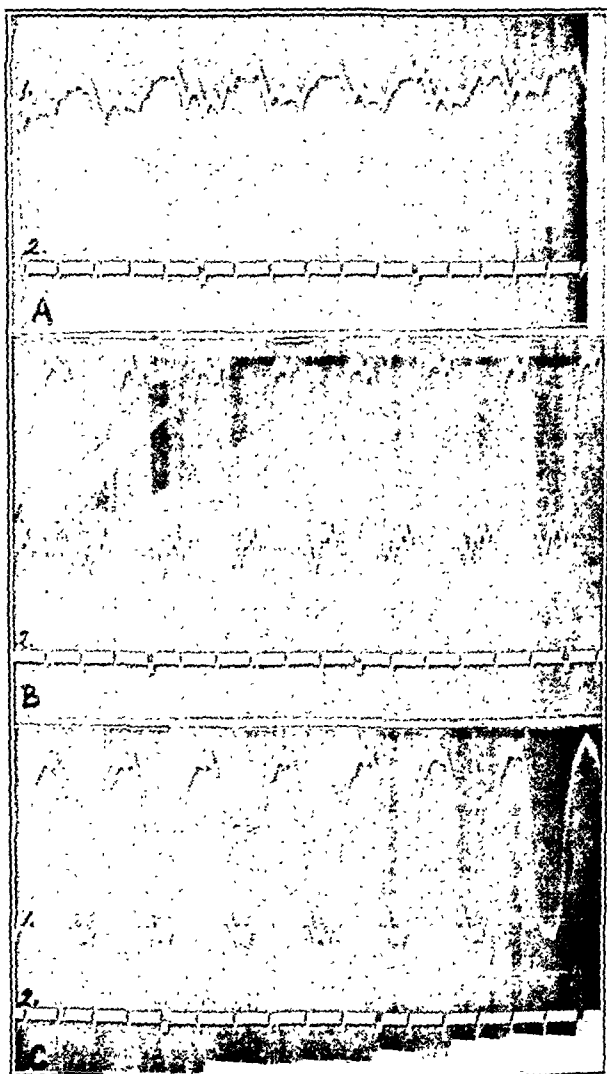


Fig. 3.—A. 1. Cardiac activity, plate over bulbus. 2. Time, one second. B. 1. Cardiac activity, plate over right auricle. 2. Time, one second. C. 1. Cardiac activity, plate over ventricle. 2. Time, one second.

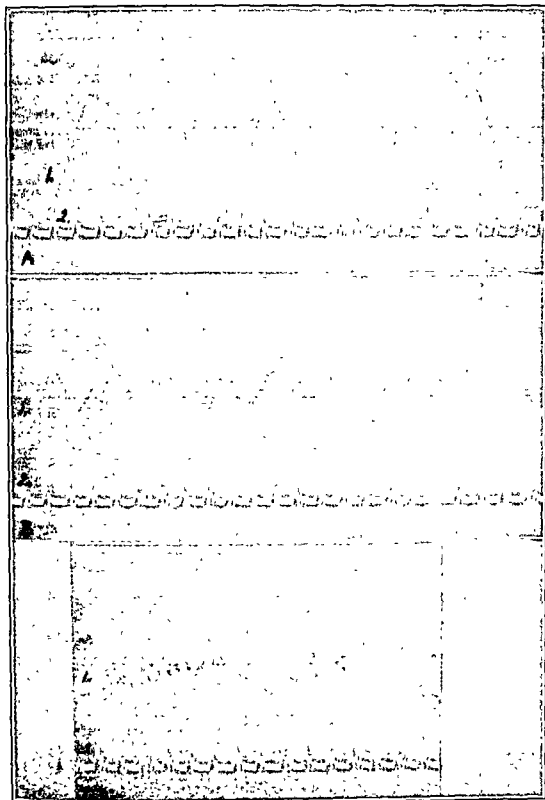


Fig. 4.—*A*, *B*, and *C* are recordings obtained from a sleeping infant, three months of age. *A*. 1. Abdominal respiration. 2. Time, one-fifth second. *B*. 1. Apex beat and thoracic respiration. 2. Time, one-fifth second. *C*. 1. Fontanel pulsations. 2. Time, one-fifth second.

Figs. 3*A*, *B*, and *C* are typical recordings obtained by placing the 3 mm. plate over various regions of the exposed three-chambered heart (turtle). With proper manipulation of the plate, any one of the displacement components may be minimized or emphasized.

Figs. 4*A*, *B*, and *C* represent curves obtained from a sleeping infant by placing the "pick up" condenser plate over the abdomen, thorax, and anterior fontanel.

Fig. 5 illustrates the appearance of curves obtained from an adult by placing the plate over the external jugular vein. The general characteristics of the fundamental venous pulse curve can be recognized. These components are marked in the conventional manner.

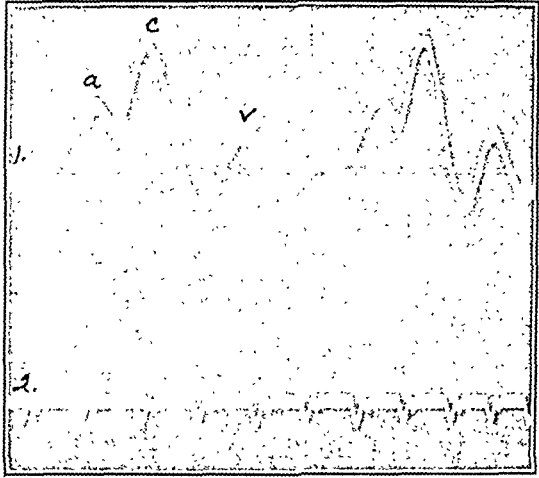


Fig. 5.—1. Venous pulse. 2. Time, one-fifth second.

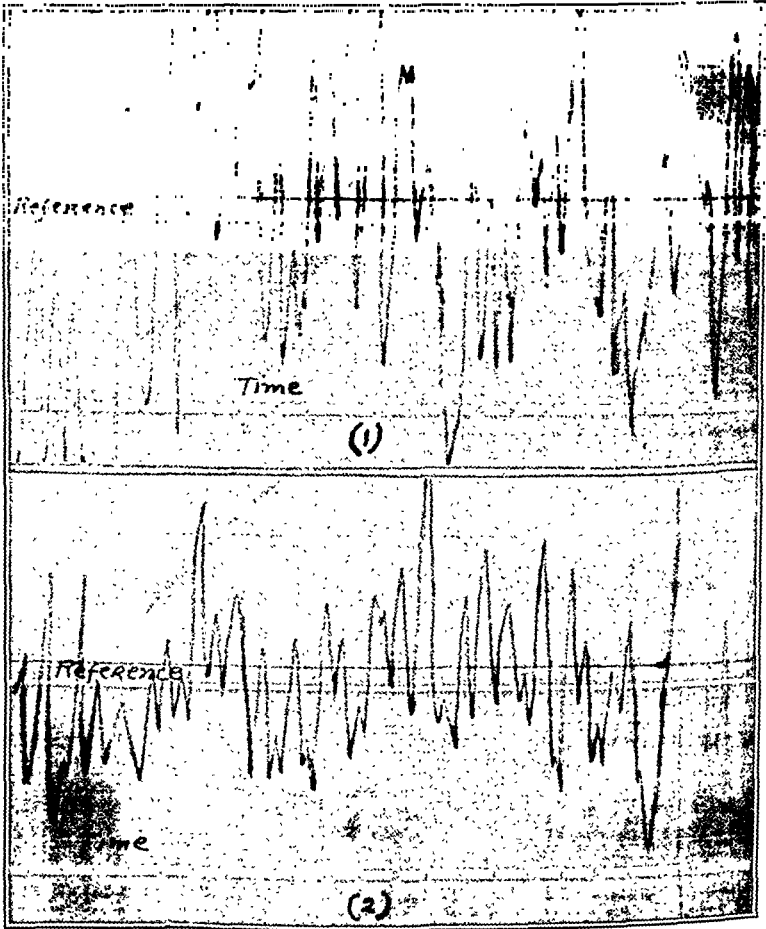


Fig. 6.—1 and 2. Time, one second.

Fig. 6 shows a possible use of the instrument for the analysis of tremors: in this case, normal hand tremors. An adult male student endeavored, with the best of his ability, to maintain the outstretched hand at a fixed distance over the plate. The subject judged the fixed distance by observing the position of a beam of light relative to a fixed point on the wall: (1) represents his ability after a number of trials, (2) represents his ability ten minutes after the ingestion of 10 c.c. of absolute ethyl alcohol diluted to 50 c.c. with fruit juice.

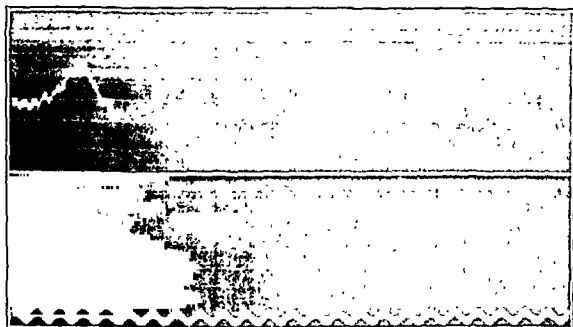


Fig. 7.—1. Kidney volume recording. 2. Time, one second.

Fig. 7 illustrates a curve indicative of spontaneous volume changes occurring in the kidney of the conscious dog. By special application an insulated plate was placed at a fixed distance from the surface of the right kidney. The animal was allowed to recover, and the recording was made seventy-two hours after the operation. Arterial pulse and respiratory and slower volume components are present.

#### CONCLUSIONS

The oscillato-capacigraph has been found valuable in making physiologic studies of surface changes. The instrument may be adapted to many uses. It is hoped that in the future other investigators will make use of it wherever possible so that its ultimate value may be established.

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# A NOMOGRAM FOR DETERMINING THE STATISTICAL SIGNIFICANCE AND THE PROBABLE ERROR OF DIFFERENCES OF PERCENTAGES\*

ROBERT SCHREK, M.D., HINES, ILL.

THE clinician, the experimenter, and the statistician frequently resort to percentages to express their findings in regard to deaths, cures, recurrences, metastasis, incidence of disease, etc. If the percentages of two series differ, it is necessary to show that the differences are sufficiently marked to be statistically significant before one can state that the experimental series differs from the control.

The significance of differences of percentages can be determined by use of various statistical formulas. The simplest one† is

$$\chi^2 = \frac{(ad - bc)^2 (a + b + c + d)}{(a + b) (c + d) (a + c) (b + d)}$$

where  $a$ ,  $b$ ,  $c$ , and  $d$  represent the experimental data. The determination of  $\chi^2$  is time-consuming and laborious. It seemed that it should be possible to construct a graph to obviate the calculation.

*Determination of Statistical Significance.*—Fig. 1 is a nomogram for determining whether two percentages differ significantly. One may assume a hypothetical experiment to illustrate its use. Suppose it is found that the mortality rate of a group of 100 patients treated with a serum is 40 per cent, whereas in a control group of 100 patients, 53 per cent die. Can it be said from these findings that the serum has any therapeutic value? In other words, is the 13 per cent difference statistically significant?

Fig. 2A illustrates the use of the nomogram for the solution of this particular problem. First locate point  $A$  corresponding to 40 per cent (abscissa  $p$ ) and 100 cases (ordinate  $n$ ). From point  $A$  draw a line to the origin of the graph,  $O$ , and extend it to meet the  $d$  ordinate for the difference of the percentages (13 per cent). The point of intersection of line  $OA$  and the 13 per cent ordinate is 114, according to abscissa  $c$ . Similarly, draw a line from the point 53 (abscissa  $p$ ) and 100 cases (ordinate  $n$ ), through the origin  $O$  and it will intersect the  $d$  ordinate for 13 per cent at point 117. Adding the two  $c$  determinations gives 231. If the sum is equal to or less than 200, a constant for which the percentage difference studied may be considered statistically different, the percentages are not significantly different. In this particular case, under consideration the sum 231 is

\*From the *Lancet*.  
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†Simmons, J. H.  
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tants is 200, the difference of the per-  
= 0.046). This  $P$  value is considered  
significance is desired, one can require  
than 128 (or  $P = 0.012$ ).

slightly greater than the constant 200 and, therefore, the difference between 40 and 53 per cent is not statistically significant and has to be considered within the realm of experimental error. It may be concluded that the hypothetical serum did not have any appreciable effect on mortality rates.

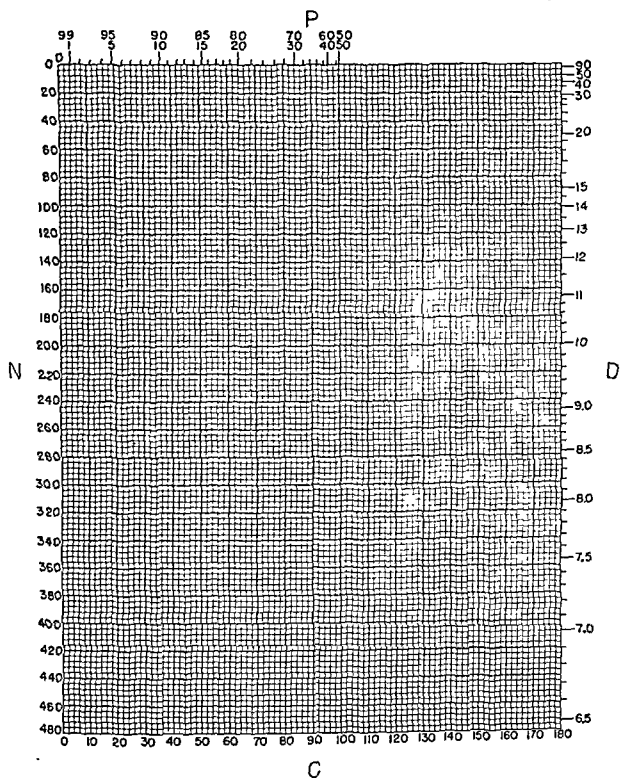


Fig. 1.—Method for using nomogram to determine the statistical significance of two percentages.

Attach a colored thread to a pin and insert pin in upper left hand corner of the graph (point O, O). With the thread taut, extend it through the graph (using the left hand ordinate  $n$ ) a upper or lower figures of the upper abscissa  $p$ ) the line representing the difference of the two percentages. Read the probable constant (using the lower abscissa  $c$ ). Similarly, determine a second probability  $c$  percentage. Add the two constants. If this sum is significant.

$N$ , number of cases;  $P$ , percentage;  $D$ , difference of percentages;  $C$ , probable constant.

In practice it is, of course, not necessary to draw the various lines. A thread attached to a pin inserted in point  $O$  can be used to determine the direction of the lines and the  $c$  constants.

It is surprising to note that, according to the graph, a difference between 70 per cent and 83 per cent in series of 100 each is statistically significant

(sum of  $c$  constants is 171), while the difference between 40 per cent and 53 per cent is not (sum of  $c$  constants is 231), although both pairs of series have differences of 13 per cent. Checking by statistical formulas and tables shows that  $P$  in the first instance is 0.03, which is considered significant, whereas in the second instance  $P$  is 0.07, which is not significant. The graph and the statistical formula are in agreement. Common sense could not be depended upon in these cases to tell whether the differences in the percentages are significant.

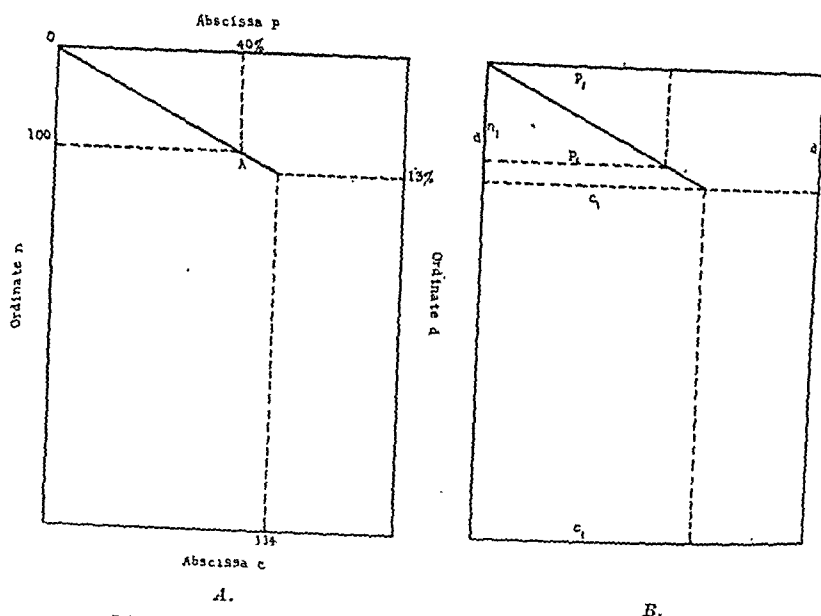


Fig. 2.—A and B show use of the nomogram. See text.

**Determination of  $P$ .**—For routine purposes the determination of the significance of the differences of two percentages suffices. In some cases it is desirable to determine the probable error or the value of  $P$ . These quantities can be readily determined from the sum of the  $c$  constants by use of Table I. The table shows that if the sum of  $c$  constants is 200 or less,  $P = 0.046$  or less. It is customary to consider values having a  $P$  of 0.046 or less as statistically significant.

TABLE I  
DETERMINATION OF  $P$  FROM THE SUM OF THE  $c$  CONSTANTS, AS OBTAINED FROM THE NOMOGRAM

SUM OF $c$ CONSTANTS	VALUE OF $P$
50	0.000063
75	0.0011
100	0.0047
125	0.0114
150	0.0143
175	0.0325
200	0.0455
250	0.0736
300	0.1024

**Proof and the Method of Construction of the Nomogram.**—For those who may be interested in the construction of the nomogram, the following brief

mathematical analysis is appended. Fig. 2B represents the nomogram and the determination of the significance of the difference,  $D$ , between two percentages  $P_1$  and  $P_2$  based on  $N_1$  and  $N_2$  number of cases. The capital letters  $D$ ,  $P_1$ ,  $P_2$ ,  $N_1$ , and  $N_2$  represent the actual figures (as 13, 40, and 53 per cent, and 100 and 100 cases, respectively, in the specific example mentioned previously), and the small letters  $d$ ,  $p_1$ ,  $p_2$ ,  $n_1$ , and  $n_2$  represent the quantities in arbitrary units. If these units are determined, the nomogram can be constructed.

Considering the similar triangles in Fig. 2B, it is seen that

$$(1) \frac{p_1}{n_1} = \frac{c_1}{d}$$

Similarly, it can be shown that

$$\frac{p_2}{n_2} = \frac{c_2}{d}$$

then, by addition

$$(2) \frac{p_1}{n_1} + \frac{p_2}{n_2} = \frac{c_1 + c_2}{d} = \frac{c}{d}$$

Let  $u$ ,  $v$ , and  $w$  be arbitrary constants of construction

and let

$$p_1 = u P_1 (100 - P_1)$$

$$p_2 = u P_2 (100 - P_2)$$

$$n_1 = v N_1$$

$$n_2 = v N_2$$

$$d = \frac{w v}{u D^2}$$

Substituting in (2)

$$(3) \frac{u}{v} \left( \frac{P_1 (100 - P_1)}{N_1} \right) + \frac{u}{v} \left( \frac{P_2 (100 - P_2)}{N_2} \right) = c \frac{uD^2}{wv}$$

Solving for  $D$

$$(4) D = \sqrt{\frac{w}{c}} \sqrt{\frac{P_1 (100 - P_1)}{N_1} + \frac{P_2 (100 - P_2)}{N_2}}$$

According to statistics

$$(5) S = \sqrt{\frac{P (100 - P)}{N}} \quad \text{where}$$

$S$  is the standard deviation of percentage  $P$

Then

$$(6) S_1^2 = \frac{P_1 (100 - P_1)}{N_1} \quad \text{and} \quad S_2^2 = \frac{P_2 (100 - P_2)}{N_2}$$

Therefore

$$(7) D = \sqrt{\frac{w}{c}} \sqrt{S_1^2 + S_2^2}$$

According to statistics

(8)  $S_D = \sqrt{S_1^2 + S_2^2}$  where  $S_D$  is the standard deviation of the difference  $D$ . Dividing (7) by (8)

$$(9) \frac{D}{S_D} = \sqrt{\frac{w}{c}} \quad \text{and} \quad c = w \left( \frac{S_D}{D} \right)^2$$



Assume that difference  $D$  is significant if  $D = 2$  times its standard deviation, i.e.,

$$\text{if } \frac{S_D}{D} = \frac{1}{2} \quad \text{or } c = w \left( \frac{1}{2} \right)^2 = \frac{w}{4}$$

It is to be concluded, then, that two percentages,  $P_1$  and  $P_2$ , differ significantly if the nomogram shows that  $c$  is equal to or is less than  $\frac{w}{4}$ , a constant (Q.E.D.).

For construction of the nomogram these formulas suffice:

$$p = u P (100 - P)$$

$$n = v N$$

$$d = \frac{w v}{u D^2}$$

Fig. 1 was constructed on 2 mm. graph paper with

$u = 0.04$  mm.,  $v = 0.5$  mm., and  $w = 800$  mm.

#### SUMMARY

Percentages or rates cannot be said to differ appreciably until they are shown to have significant differences.

The statistical significance of differences of percentages can be calculated by formulas, but this method is time-consuming and laborious.

By means of the nomogram it is possible to determine whether two percentages have significant differences. The nomogram can also be used to find the  $P$  value of the difference of two percentages.

### A SIMPLIFIED APPARATUS FOR THE PRESERVATION OF BACTERIAL CULTURES IN THE DRIED STATE\*

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**D**URING an intensive bacteriologic study of the stools of infants and young children with acute enteritis it became desirable to have at our disposal a simple apparatus for the preservation of some of the bacteria in a viable state without the loss of virulence. immunologic or biochemical characteristics. There are numerous reports in the literature regarding the length of time bacteria survive without change after being dried from the frozen state. Swift<sup>1</sup> reported that hemolytic streptococci dried from the frozen state in 1916 to 1917 were still viable and possessed their group specific substance in 1937. Lancefield,<sup>2</sup> in her studies with hemolytic streptococci preserved by freezing and drying over a period of twelve years, observed that such cultures consistently maintained the characteristics noted at the time of their original

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preservation. Pneumococci similarly preserved maintained their type specificity. Rake<sup>3</sup> observed that meningococci maintained their original virulence and specific carbohydrate content for at least twenty months when preserved in a similar manner.

The general principle involved in these reports is that of removing water by distillation under reduced pressure from material while it is frozen. Two general methods are employed. In the one, material is previously frozen in a dry ice bath (solid carbon dioxide), attached quickly to a suction pump, and the water vapor condensed in a dry ice condenser.<sup>4</sup> In the other method, which has been developed by Flosdorf and Mudd,<sup>5,6</sup> the material is first "degassed" under somewhat reduced pressure and then subjected to very low pressure while the water vapor is absorbed by a chemical desiccant. Flosdorf and Mudd use drierite (anhydrous calcium sulfate)

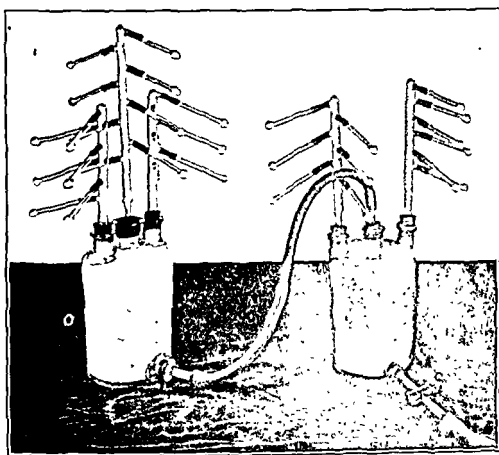


Fig. 1.—Apparatus for dehydrating small quantities of bacterial cultures from the frozen state.

We have utilized the latter method, i.e., the freezing and removal of water vapor under reduced pressure and its absorption on a chemical desiccant, in devising a simple apparatus from material available in most laboratories. The availability of the apparatus, its simplicity of construction and operation, and the economy in time, together with the satisfactory results obtained, prompt us to describe it.

#### APPARATUS

The equipment consists of a vacuum pump similar to a Hyvac pump, capable of reaching a pressure of approximately 0.2 mm. Hg; a good grade of pressure tubing; pyrex Woulff bottles of 2 liter capacity; 9 mm. pyrex tubing from which bulb containers may be easily blown; a cross-fire oxygen-gas torch for sealing the bulb containers under vacuum; a suitable desiccant,

such as drierite; incidentals, such as rubber stoppers, rubber connections, and large bore pyrex glass tubing from which the manifolds can be made. This apparatus is illustrated in Fig. 1. A spark coil vacuum tester is of much assistance in locating any very slight leak in the apparatus. It is our custom to go over the entire apparatus as soon as a good vacuum has been established, and in this way locate and eliminate any tiny leaks which, if permitted to remain, would result in slow and improper desiccation.

The bulb containers are blown from pieces of 9 mm. pyrex tubing, 8 cm. long, so that the bulb capacity is 0.5 to 0.8 c.c., facilitating drying 0.2 to 0.3 c.c. of culture. The manifold is made from 15 mm. pyrex tubing to which are attached 6 or 8 side arms of 6 mm. pyrex tubing, 2 cm. long, set at an acute angle of 70°. We dry 32 ampoules of cultures daily, using five such manifolds.

#### METHOD AND PROCEDURE

The method is essentially that of vacuum drying from the frozen state without preliminary freezing in dry ice or an ice salt bath. This method has been very well described by Flosdorf and Mudd<sup>6</sup> and for further details the reader is referred to their study. Briefly, our routine procedure is as follows: Pieces of rubber tubing, 2 cm. long, are attached to each of the side arms on the manifolds. The manifolds are then wrapped separately in paper and sterilized in the autoclave for twenty minutes at 15 pounds pressure. The Woulff bottles have previously been filled with regenerated drierite and stoppered tightly. At the time of drying, a sterile manifold is fitted into one of the openings in the Woulff bottle-desiccating chamber with a good grade of rubber stopper, preferably of gum. An eighteen-hour-old salt-free broth infusion culture of the microorganism to be preserved is mixed with an equal volume of sterile skim milk and 0.2 or 0.3 c.c. of the above mixture is added, without contamination, to the previously plugged and sterilized bulbs. Milk seems to prevent excessive bubbling during the degassing period and facilitates rapid resuspension of the dried bacteria in water whenever desired at a later date. The insertion of the manifolds in the Woulff bottles before connecting the bulbs simplifies manipulation and decreases the possibility of contamination. These bulbs are attached to the rubber connections on the manifold with the aid of an ordinary rubber tube stretcher, the width of the jaws being reduced so that touching the rubber connections by hand is unnecessary. Occasional flaming of the exposed ends of the rubber tube connections and the tongs reduces the danger of contamination. After loading all manifolds the rubber stopper connections between the manifolds and the Woulff bottles are greased, tightly inserted, and the vacuum pump started. The system is allowed to evacuate, and the stopcock between the pump and Woulff bottle is closed simultaneously with the development of the first bubble. The cultures are then allowed to degas for approximately ten minutes, at the end of which time evacuation is continued. The cultures will freeze and the containers frost over after about three to five minutes of pumping, if the degassing process is complete and the system is free from leaks. The degassing period should be extended in case bubbling continues as the cultures are being

pumped to freeze. After vacuum freezing the moisture vaporizes to the drierite with sufficient rapidity to retain the cultures in the frozen state throughout the remainder of the desiccating period which is approximately four hours.

The tubes are left under vacuum for a total of eighteen to twenty hours to insure complete drying. At the end of this period the ampoules are cut off near the rubber connection under vacuum by means of the cross-fire oxygen-gas torch. The cultures are thus sealed under vacuum in all-glass containers and are stored in the refrigerator until subcultured. According to the literature, the above conditions are most ideal for preservation of bacteria. Upon subculturing, the ampoules are marked with a file near the bulb and opened in a flame with the aid of asbestos gloves. During this procedure care should be taken to avoid heating the organisms. After opening the container, the subculture medium is introduced with a sterile, slender-tipped pipette. The desiccated materials are exceedingly hygroscopic, and there is no difficulty in obtaining a suspension of the organisms. We have had no difficulty with contamination while using the above procedure.

The drying capacity of the drierite is limited, but it may be readily regenerated by heating in an oven overnight at a temperature ranging from 160 to 200° C. A shorter regeneration period, at higher temperature, is recommended by the distributors of drierite, but for routine convenience the former conditions are found to be more satisfactory. The drying capacity of the contents of each Woulff flask may be estimated and regeneration carried out when necessary since 20 gm. of anhydrous drierite is adequate desiccant to freeze and dry 1 c.c. of culture suspension. The rubber connections are renewed at intervals, depending upon the nature of the tubing and the frequency of their use.

We have used this apparatus extensively during the past summer and have found it satisfactory for drying and preserving cultures. From August 10 to August 31 we made 12 runs and dried and preserved 354 vials of cultures. We started with 5 pounds of freshly regenerated drierite and used it throughout the 12 runs without regeneration. We find upon subculturing these dried cultures six months later that they are viable and have retained their original cultural characteristics. This study is in progress and further observations on these dried cultures are planned.

We have also found this apparatus satisfactory for concentrating and drying human blood serum from the frozen state by the Flosdorf and Mudd technique.

#### SUMMARY

A simple, inexpensive, low vacuum apparatus is described which satisfactorily freezes and dries 32 bacterial cultures daily. The small amount of time required for operation is much in its favor. The simplicity of the apparatus and procedure makes low temperature desiccation of heat labile substances available to the average laboratory.

We are indebted to Mr. Frederic E. Holmes, of the Children's Hospital Research Foundation, for blowing the glass manifolds.

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## AN AMPLIFIER FOR RECORDING HEART SOUNDS THROUGH THE USE OF THE CATHODE-RAY TUBE\*

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MANY workers who are interested in the recording and study of heart sounds make use of battery-operated amplifiers and some form of galvanometer-recording device.<sup>1-5</sup> In considering the several types of instruments in use, the conclusion may be reached that these systems have shortcomings worthy of mention. For example, batteries require replacement and constant care; they require a separate carrying case or a large single case to house both the amplifier and batteries; the voltage of the several types of batteries used is subject to continuous change, and as a result the amplifying characteristics of the amplifier are variable. Further, battery-operated tubes tend to be microphonic due to their structural characteristics. As for string or mirror galvanometers, they must be critically standardized to avoid overshooting; they may have inherent resonant periods and harmonic distortions which frequently fall directly in the range of the sound frequencies under investigation. It would appear that the medical profession has assumed the string galvanometer a suitable recording instrument, but electrical engineers do not wholly agree with this contention. Thus, Reid<sup>6</sup> indicates as two major defects of the string galvanometer: (1) Above 20 cycles per second distortion in the form of harmonics, takes place, and as a result the string does not respond equally well to high and low frequencies. (2) The presence of body resistances of about 2,500 ohms in the string circuit will cause the string to react in a manner not easily controlled or compensated for.

Recognition of the importance of these factors, coincident with a study of electronic devices for more accurately recording heart sounds, has led to

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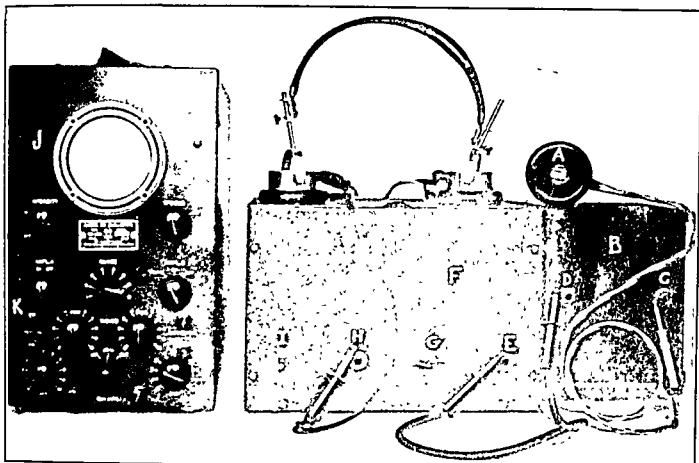


Fig. 1.—The cathode-ray stethograph, less camera. A, crystal microphone. B, preamplifier. C, input; D, preamplifier output to E or K; E, amplifier input. F, main amplifier; G, volume control; H, headphone output; I, line switch; J, viewing screen. K, oscillograph input.

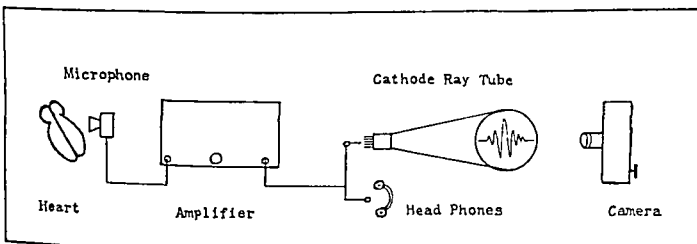


Fig. 2.—Pictorial representation of apparatus used.

the development of an A.C. operated amplifier which can be used either as an electrical stethoscope, or, in conjunction with a cathode-ray tube, as a cathode-ray stethoscope. A photograph of the apparatus which has been developed is shown in Fig. 1. The sketch given in Fig. 2 presents the arrangement of the several components of the instruments used in the heart sound studies. The microphone used is of the piezoelectric type, that is, one containing a magnesium sulfate crystal which produces minute voltages when sound waves impinge upon its surface. The resulting voltage varies in accordance with the amplitude and frequency of the sound wave and is impressed on the grid of the first tube of the amplifier. This type of microphone is small, light, and sturdy, and may be used in any position. Its frequency response is excellent; it possesses no hiss or background noise, and it requires no matching transformers or batteries.

The amplifier has been designed to have high gain for frequencies as low as 30 cycles per second and to dampen frequencies higher than 1,000 cycles per second. This provision in the design of the instrument has been included since heart sounds usually have a frequency range of from approximately 40 to 200 cycles. Thus, with the unnecessary higher frequencies damped out, a major cause of interference is eliminated. The tubes used are of the metal type. The power supply is taken from any common wall source of 110 volts, A.C. The power transformer is placed within the main cabinet which also includes the rectifier tube, an efficient filter circuit, and two amplifying tubes. Inspection of the photograph presented in Fig. 1 shows a preamplifier, a small metal box attached on the right of the larger box. This unit consists of a two tube circuit of particularly high gain and sensitivity,

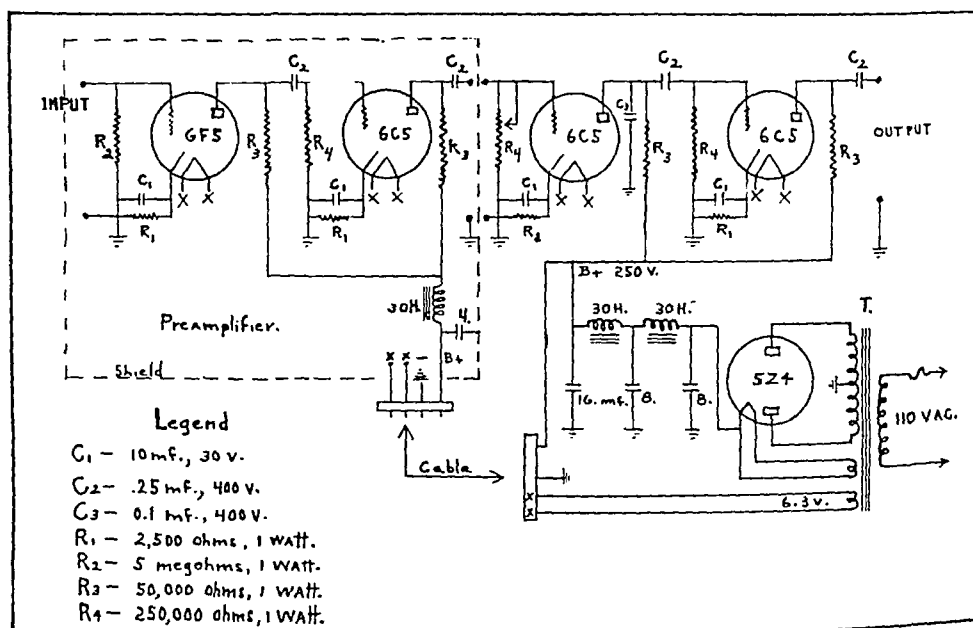


Fig. 3.—Schematic diagram of amplifier.

a characteristic necessary for adequate amplification of the low output of the crystal microphone before it is introduced into the main amplifier. On the front of cabinets B and F in Fig. 1 may be seen four holes to receive the four plug-in jacks. From right to left the holes are microphone input, preamplifier output, main amplifier input, and headphone output. The connecting cable with jacks is used between the two units when all tubes are desired in the circuit. The preamplifier is the condenser output and may be used to actuate the galvanometer on an electrocardiograph or the cathode-ray oscillograph seen in Fig. 1.

The schematic diagram of the entire amplifier is given in Fig. 3. It may be noted that the preamplifier is shielded from the chassis containing the power transformer, and that the B-plus supply has additional filtering within the preamplifier. These two conditions contribute greatly to making the amplifier quiet and hum free.

With the headphones plugged into the preamplifier alone, the volume of the heart sounds is about equal to that of the ordinary stethoscope. With the entire amplifier in use, the volume control needs to be advanced less than half its extent to obtain more than sufficient volume for the headphones. For the purpose of recording, the output of the preamplifier is impressed directly on the input of the cathode-ray oscillograph. If a string or mirror galvanometer is employed, the preamplifier output is introduced directly into the string circuit.

The cathode-ray tube is not a new device, since, in fact, it preceded the development of the ordinary radio tube.<sup>7</sup> It may be traced back to the famous Geissler tube from which was developed the Crookes tube. In 1897 Dr. F. Braun, a German physicist, modified the Crookes tube to make the first cathode-ray tube. He did this by placing a metal shield with a hole in it in the path of the electrons passing toward the target or screen. Consequently, instead of a cloud of electrons proceeding to the target end of the tube, only a pencil of electrons passes through the hole in the shield. In this way, a method of focusing and varying the size of electron beam was attained. Further experimentation demonstrated that this beam, when striking certain substances, was capable of causing them to fluoresce. Previously, it had been discovered that a beam of electrons could be bent to one side or the other by electrical or magnetic influence. Braun and his co-workers combined his innovation with the previous discoveries to produce the modern cathode-ray tube.

The cathode-ray tube thus has three essentials: the pencil of electrons, a fluorescent target for this pencil to write on, and some method of bending the beam in response to whatever changes it is desired to measure. These essentials of the tube have not been changed since Braun's time, but many significant improvements have been made in details, such as making the electron pencil sharper and more responsive, improving the fluorescent screens, lowering the operating voltages, and lengthening the tube's life.

In referring to Fig. 2 it may be seen that the modern tube is flask-shaped, the electrons are produced in the narrow neck of the tube by a heated filament, and the beam strikes the base or target and traces visibly the phenomena being studied. The photograph in Fig. 1 shows a modern cathode-ray unit with the various controls for focus, intensity, frequency, and amplitude of the beam.

The tube has many applications<sup>8, 9</sup> and is used throughout the industrial world, in television, in aviation for blind flying, and, more recently, in the medical world. In connection with the last application, it is used in several physiology laboratories for the study of Berger's wave of the brain, muscle and nerve responses, registration of the heart sounds, and in electrocardiography. Concerning this last use, Frey<sup>10</sup> has stated that the cathode-ray oscillograph is superior to moving coil oscillographs, chiefly because it will register accurately at all vibration frequencies present in carrying out a study of the heart.

Experience with the visual registration of heart sounds on the screen of the tube reveals that the wave forms may be observed instantly and accurately.





Fig. 4.—Heart sound tracing from cathode-ray tube. Film speed 200 mm. per second.

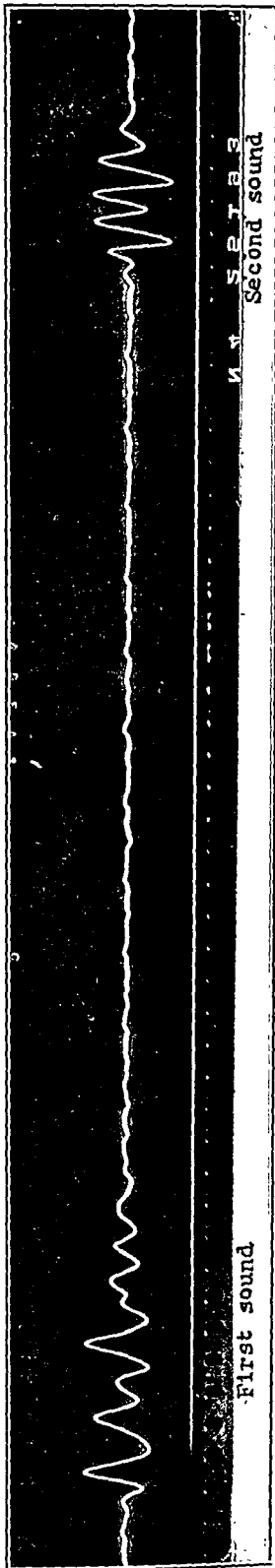


Fig. 5.—Tracing similar to above but with film speed of 400 mm. per second.

and with any desired amplitude. Although disturbances of rhythm, rate, and sounds can be seen on the screen, they are in essence transitory and pass rapidly. In this respect, visual observations of heart sounds, without provision for permanent recording, assume the same shortcomings which are inherent in the use of the stethoscope. For this reason graphic recording is essential for detailed study of the sound events in the cardiac cycle. As shown in Fig. 2, a recording camera using a moving film was necessary to photograph the movement of the electronic pencil on the target.

The sample tracings of heart sounds shown in Figs. 4 and 5 were photographed from the screen of a blue spot, short persistence cathode-ray tube using a running film camera having an  $f/2.5$  lens. The film used was 35 mm. supersensitive panchromatic.

In Fig. 4 a heart sound tracing is shown illustrating two cardiac cycles. The film speed was approximately 200 mm. per second. The base line can be seen to have present a faint 60 cycle ripple, the cause of which was the lack of adequate shielding of the tube at the time the tracing was taken. A slight halo accompanies the recording spot, this being due to the glow of adjacent particles of the fluorescent material of the tube screen. Fig. 5 illustrates a single heart sound cycle from the same patient but with a film speed advanced to 400 mm. per second. At this speed the spot halo is not as noticeable. The speed of 200 or 400 mm. per second is far greater than is necessary for routine stethograph studies, but has been reproduced here to demonstrate the practicability of this method of recording. The optimum speed for heart sound recording is probably 100 mm. per second.

#### SUMMARY

A description is given of a heart sound amplifier which uses a cathode-ray tube as a recording device for reproducing in the form of photographic tracings the sound patterns emanating from the functioning heart.

The author is indebted to W. C. Mock, Jr., of the U. S. Bureau of Standards, for assistance in producing the cathode-ray tracings in Figs. 4 and 5.

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# THE EAGLE COMPLEMENT FIXATION TEST FOR SYPHILIS

## A NOTE ON THE AMBOCEPTOR TITRATION

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WITH the increasing use of the complement fixation test for syphilis, as outlined by Eagle,<sup>1</sup> it seems likely that some will encounter certain difficulties met in our laboratories at the time of our first experiments with the technique in October, 1937. For that reason, it may be well to detail the major difficulty, and the means by which it has been satisfactorily overcome.

In our hands, the test, run in parallel with an eighteen-hour fixation and both Kahn and Eagle flocculation tests, proved at first definitely inferior in sensitivity. At the same time, complement titrations set up with the Eagle fixation test indicated the presence in the reaction tubes of considerably more than the  $2\frac{1}{2}$  units of complement required. In reviewing the theoretical considerations of the test,<sup>2</sup> it was found that in an effort to secure  $2\frac{1}{2}$  units of complement in the test proper, use is made of the supposed approximately reciprocal relationship between amboceptor and complement, and the amboceptor is titrated daily, using  $2\frac{1}{2}$  units of amboceptor, thus determined, to sensitize the sheep erythrocytes for the test. It was apparent from the data that, either because of unusually potent complement or some similar factor, the concentrations used were outside the range in which the relationships between amboceptor and complement are even approximately reciprocal.

However, it seemed advantageous to retain, if possible, the principle of daily amboceptor titrations, since as Eagle has pointed out, and as data previously secured had confirmed, the day-to-day variation in resistance of sheep erythrocytes to hemolysis, as measured by titration with amboceptor, is much greater than the day-to-day variation in the complementary activity of fresh guinea pig serum secured from a fairly large pool of carefully selected animals. It appears logical to assume that the daily titration with amboceptor may be retained, and control of complement concentration simultaneously achieved, if the amboceptor titration be set up with complement two-fifths the concentration of that employed in the test, and the unit of amboceptor so secured be used to sensitize the sheep erythrocytes. The test proper must then of necessity contain  $2\frac{1}{2}$  units of complement per tube.

In practice, the tests are first set up, precisely as outlined by Eagle<sup>1</sup> with complement diluted 1:10, after which a portion of the residual complement is further diluted to give a final dilution of 1:25 (1.4 ml. of 1:10 complement added to 2.1 ml. of isotonic saline suffices for seven tubes), and the 1:25 dilution is used in the amboceptor titration, which in all other respects is carried out in accordance with the standard technique. The highest dilution of amboceptor which gives complete hemolysis in thirty minutes is then used to sensitize the sheep cells (and not, let it be pointed out,  $2\frac{1}{2}$  times this amount, as with the original method).

With this minor modification, the Eagle complement fixation test for syphilis has, in a series of more than 10,000 cases, given a high degree of sensitivity, with a specificity rating certainly in excess of the requirements of the United States Public Health Service.

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## ILLUMINATING BOX FOR FLOCCULATION (KAHN) AND SEDIMENTATION TESTS

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THE satisfactory performance of any flocculation or sedimentation tests depends upon the proper illumination of the test tubes. Reliance upon daylight, especially during the winter months, and upon ordinary electric light, is not always satisfactory.

The construction of an illuminating box, which will permit the passage of light through the sides of the test tubes, as well as from below through the depth of the fluid, helps to solve this difficulty. In order to have the light pass from beneath, it is necessary that the lower cross support of the test tube rack be replaced by a piece of clear glass, and the rack be painted with a dull black paint to prevent reflected light.

The box shown in Fig. 1 is made of galvanized sheet iron, the size of the box depending upon the width of the test tube rack. A convenient size which will accommodate the ordinary test tube rack is 13 inches wide, 10 inches high, and 7 inches deep. A small recessed platform is made to extend across the front of the box, and it is upon this platform that the test tube rack is placed.

The base and back of this platform are made of two panels of daylight blue flashed opal transilluminating glass which may be obtained from any x-ray supply house.

The interior of the box is painted with a flat white paint; the exterior with a flat black paint. On the posterior wall of the box, at the top and bottom are two rows of half inch holes to permit ventilation and for the passage of electric wire from the lamps to the socket.

For illumination two 60 watt frosted electric light bulbs are placed, one at either end of the upper portion of the box, so that the light is reflected downward, and through the two panels of glass. The top of the box is fitted with a cover to permit replacement of the bulbs when burned out (Fig. 2).

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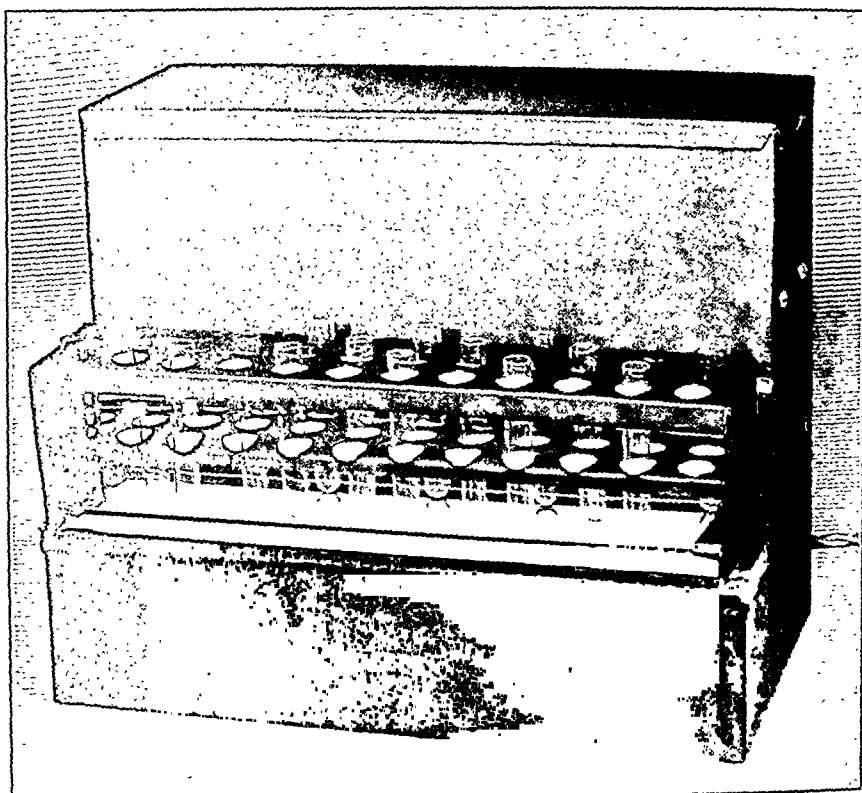


Fig. 1.

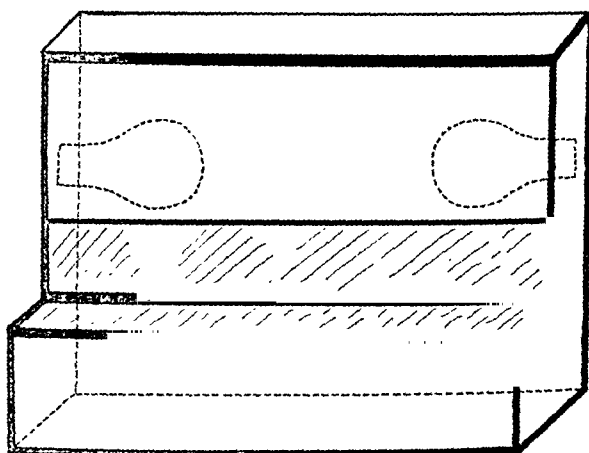


Fig. 2.

It is found of advantage to have a narrow strip of black paper glued to the bottom of the upright panel of glass to permit a break in the area of light passing through glazed area. The box should be made light enough to permit easy handling, so that when the test tube is in position on the platform, the whole apparatus may be lifted to level of the eye and viewed.

The cost of making the box by a tinsmith, including paint, electric bulbs, sockets, wiring and other incidentals was less than five dollars.

## THE SEALING OF MUSEUM JARS\*

M. H. BOOK, M.D., AND M. W. SULLIVAN, M.D., BOSTON, MASS.

TWO devices which have been of value in the sealing of flat-top museum jars are described here. They are particularly useful to those who do not have enough of this work to acquire the proficiency of experts.

The cement used depends on several considerations, but long experience has shown that the best preparation is one composed principally of asphalt. Formulas are given by Little<sup>1</sup> and Mallory.<sup>2</sup> We have been using a battery sealing compound† with satisfactory results. Most other cements are open to the objection that they are dissolved by one or more of the reagents used in the preparation of the specimen.

The gas-heated cement applicator shown in Fig. 1 enables one to apply a neat ribbon of the softened cement to the edge of the jar in a short time. The container for the cement is made of two pieces of tin, soldered together with high melting point solder in the shape of a scoop about 6 cm. long. The open end is pinched together with pliers to leave an opening 1 mm. wide. The back of the scoop is screwed to the end of a wooden handle, such as is used in chisels. The bottom of the handle is grooved to take the gas line, bent in the shape shown. A blowpipe makes a satisfactory gas line. Staples are used to fasten it to the handle, but it should be left just loose enough so that its position with respect to the scoop may be altered if necessary.

A good method of affixing the top in order to obtain a perfect seal is to heat it when it is in the proper position and apply pressure evenly. An ordinary household electric iron is useful for this purpose, but for large jars it is desirable to have a heater with a larger surface. The heater shown in Fig. 2 was made for this purpose. Into a cake tin 7 by 11 by 1¼ inches is poured 5 pounds of refractory cement,‡ mixed with water. In this is embedded a 660 watt replacement heating coil, which may be obtained from any hardware store. The ends are attached by bolts to a length of asbestos-covered electric wire. A wooden handle fastened by wires to the ends of the pan completes the device.

The actual procedure in sealing a jar is as follows: The mounted specimen is carefully placed in the jar, and the preserving fluid is added to within 2 cm. of the top edge. The latter, as well as the lid, should be scrupulously clean and dry. With the applicator a ribbon of the melted cement is deposited on the edge of the jar, an attempt being made to keep to the outer

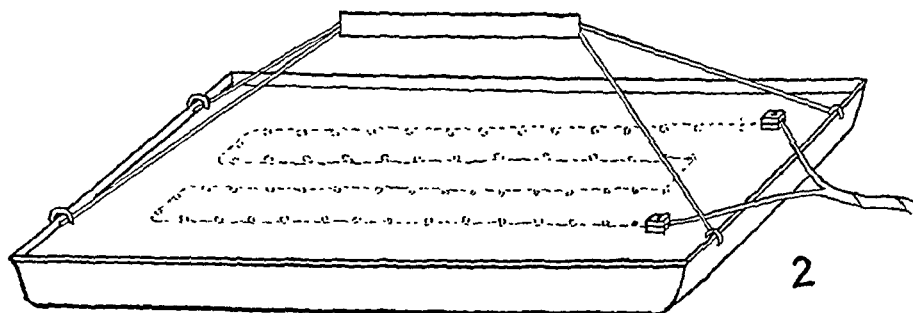
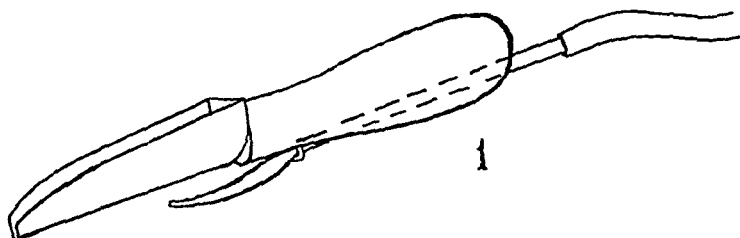
\*From the Department of Anatomy, Tufts College Medical School, Boston.

Received for publication, March 11, 1959

†RP Battery Sealing Compound No. 359, manufactured by the H. H. Robertson Co., Pittsburgh, Pa.

‡Alundum refractory cement RA 102, obtainable from the Central Scientific Co., Cambridge, Mass.

surface. The lid (which must have a small hole in the back left-hand corner) is placed in position, and the electric iron or heater, at room temperature, on top. The current is then turned on, and as the lid warms up it softens the cement. When a broad seal all the way around is obtained, the heater is removed. Care should be exercised not to have the seal too thin, otherwise it may be easily broken by jarring or temperature changes. When the cement



is thoroughly cooled, preservative fluid is added through the vent with a needle and syringe to 1 cm. from the top. It is not wise to have the air cushion over the fluid too thin, as a period of hot weather might break the seal of the vent. The latter is closed with a drop of hot cement. Excess cement is scraped off, and the jar is cleaned with gasoline. The seal may be made more attractive by applying a narrow border of black enamel, using masking tape to obtain a straight edge.

No matter how carefully the jar has been sealed, it should be examined from time to time, as temperature changes occasionally cause a break in the seal, especially at the vent.

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## AN INSTRUMENT FOR OBTAINING BONE MARROW\*†

G. O. FAVORITE, M.D., PHILADELPHIA, PA.

THE trephine and the needle puncture are the current popular methods for obtaining bone marrow for study. The sternum is the usual source of the marrow, and the present methods are more or less designed for this bone. The needle biopsy is easily performed, but the material obtained usually consists of a mixture of blood and marrow cells. Quite often the lack of cellularity in the preparation does not represent the true state of the marrow. On the other hand, the trephine method often requires the services of the surgeon. As a diagnostic procedure it must be admitted that it is formidable, and, at times, out of proportion to the information obtained. The disadvantages are outweighed by the cellularity of the preparations and material obtained for histologic sections in studying the topography of the marrow. An excellent critique of the comparative methods is given by Dameshek, Henstell, and Valentine.<sup>1</sup> They conclude, in part, that the trephine method is far superior to the puncture technique.

It seemed that there should be a compromise between these two extremes, that is, ease of performance with poor cellularity of the needle method, and comparative difficulty of performance with good cellularity and topography of marrow with the trephine. With this thought in mind, an instrument has been devised which seems to strike a happy medium. In no way is it intended to replace the trephine. On the other hand, it has been found far superior to the needle method since actual marrow is obtained for smears, and in many instances, for histologic sections.

The instrument consists of a No. 11 gauge cannula, a trocar, and a small drill which fits the cannula, as shown in Fig. 1. The cannula and trocar have been used for some years by our laboratory in bleeding donors. The drill is fitted with locking nuts which regulate the extension of the drill beyond the cannula. The tip has shallow cross grooves which aid in retaining the marrow. In the adult of average skeletal development, this distance is 4 mm.

The gladiolus is the site of operation. The most desirable location is the level of the third or fourth intercostal space. This region is especially desirable in women for cosmetic reasons as well as for the cellularity of the marrow.

The technique of operation is easily acquired after several performances. After sterilization of the surface with alcohol, the skin, soft tissues, and periosteum are infiltrated with a local anesthetic, such as 1 per cent novocain. The periosteum is infiltrated for an area of 1 cm. in diameter. If the infiltration is adequate, the patient experiences no pain. The cannula, with the trocar in place, is introduced obliquely through the skin, in the direction of the long axis of the sternum. Since the caliber of the cannula is rather large,

\*From the Clinical Laboratories, Hahnemann Hospital, Philadelphia.

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†The instrument is manufactured by the Scientific Equipment Co., Philadelphia.



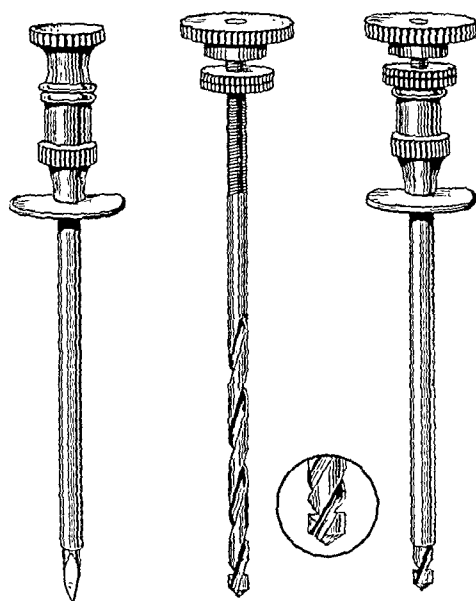


Fig. 1.—From left to right, the figure shows the cannula and trocar, the drill with the details of the tip in the circle, and the drill within the cannula.

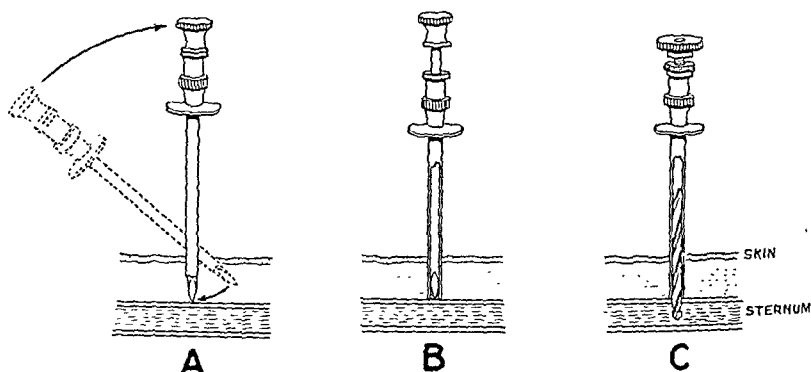


Fig. 2.—A. The dotted figure shows the direction for introducing the cannula and trocar through the skin. When this maneuver is completed, the instrument is rotated in the upright position. B. The cannula is pushed downward to rest on the sternum. C. The drill has penetrated the sternal plate and the tip is within the marrow cavity.

the skin must be fixed firmly against the chest wall with one hand while the cannula is penetrating the skin. If this is not done, the skin will buckle, and considerable force is then required to penetrate it. It is essential that the cannula itself, regardless of the trocar, must enter the skin for a distance of at least 0.5 cm. The cannula and trocar are then straightened in the upright position, as shown in Fig. 2A. The trocar should touch the periosteum in the center of the anesthetized area. With the trocar as a guide, the cannula is forced downward so as to rest on the periosteum. This maneuver is illustrated in Fig. 2B.

The trocar is removed, and the drill is introduced in the cannula, which now serves as a guide and as a guard. It also determines the depth to which the drill will enter the sternum. One must ascertain previously that the locking nuts are firmly tightened at the proper distance. The drill is operated by

firmly turning and applying a varying amount of force downward until it catches. Thereafter it will proceed downward by merely rotating it. When the drill has reached its full predetermined depth (Fig. 2C), it is further rotated several times, and then withdrawn while the cannula is held firmly in place. The marrow will be found in the grooves of the drill. With the point of a needle (gauge 19) the fragments can be removed and smears made on clean slides by gently streaking the marrow on the glass surface. The bevel of the needle will hold the fragments in position for this purpose.

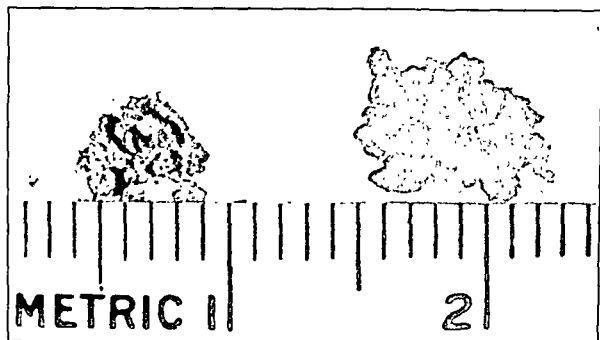


Fig. 3.—A photograph showing the amount of marrow material obtained in two cases.

The drill should be reintroduced several times. It is essential that the cannula be held firmly against the sternum in the upright position so that the original hole is not lost. By varying slightly the direction of the cannula and drill, additional marrow is obtained for histologic sections. Fig. 3 represents the average amount of material obtained in two of the 50 cases in which the instrument has been used.

The cannula is withdrawn in the usual manner. There is left a small puncture wound of the skin. This can be minimized by pressing it between the fingers, thus flattening and approximating the edges. It is then covered with a dry sterile dressing.

In conclusion, we feel that this instrument, essentially a drill, is a worthy compromise between a needle and a trephine. It offers all the advantages of the needle and most of those of the trephine. The cellularity of the smears is similar to the trephine preparations. Small marrow fragments often give a fair topography of the marrow but do not compare with the large sections obtained with the trephine. The method can be performed at the bedside and can be repeated many times.

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## ADAPTATION OF THE ROLLS RAZOR TO A NEW TYPE OF MICROTOME BLADE\*

J. M. FEDER, M.D., ANDERSON, S. C.

THERE exists a palpable need for an auxiliary microtome blade in the larger laboratories, an all-purpose blade in small laboratories and an inexpensive one to be used by students in their first attempt at microtomy.

The Rolls blade, held in a suitable holder, meets most of the requirements of each group. When so employed, it can be used in connection with all types of microtomes, sliding, rotary, or "swing." It works equally well with paraffin or frozen sections, and as a consequence, can be instantly changed from one type of service to another.

The orthodox microtome blade is a dangerous weapon in the hands of beginners, and the blade described greatly minimizes the risk of personal injury by the inexperienced worker.

For the past two years an attempt has been made by us to evaluate all of the various blades now in use in an effort to select one that would most nearly meet the major requirements for a universal edge. The Rolls razor was selected for the following reasons:

1. The Rolls blade has approximately the same degree of hardness as the finest microtome blade and is made from the very best English steel.

2. The blade nearly approaches in shape the ordinary microtome knife. (Rolls razors are hollow ground; however, through the cooperation of the makers, blades purchased for this purpose will be "chisel ground" if requested. When sharpened in this manner, they are much more satisfactory.)

3. The unique construction of this razor makes honing and stropping easy for the most inexperienced in this otherwise difficult procedure. One lid of the box contains a hone, while the other contains a fine strop. The blade is attached to a sliding mechanism by a half turn, and the stropping handle moved backward and forward. The edge is brought into contact with strop and hone at the proper angle and is automatically reversed when the maximum excursion has been reached.

This ease of stropping and honing assures a keen-edged knife at all times, and the facility with which the blades can be stored aids in keeping them in a safe place when not in use.

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\*From the Laboratories of Anderson County Hospital, Anderson.  
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*Holder for Rolls Blades.*—A piece of tool steel, 12.5 cm. in length and 0.5 cm. in thickness, was tapered down toward a blunt edge, approximately as nearly as possible the shape and size of an ordinary microtome blade. The holder measured 2.9 cm. in breadth, and at the thinnest portion measured 0.2 cm.

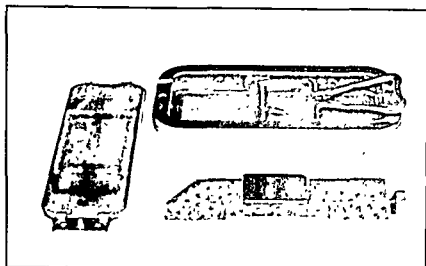


Fig. 1.—Holder with blade in position. Above, holder, stropping and honing appliance is shown. Hone is in position for use, and strop is shown at left. By the expedient of placing strop on box and removing that part containing hone, the situation is reversed, and stropping can be carried out.

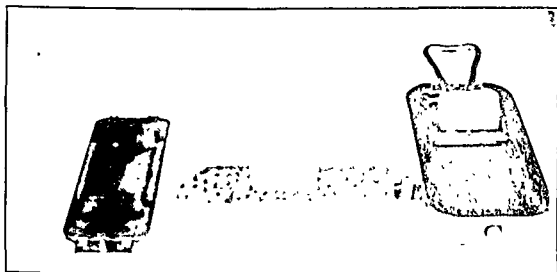


Fig. 2.—Holder with blade detached. The blade is in sharpening element ready for honing. It can be attached and removed by a simple half turn.

In the central portion of this bar of steel, a slot was cut to accommodate the blade. This opening is 3.8 cm. long and 1.5 cm. deep. On the left end of this opening, two pins were placed to engage in the two depressions noted on one end of a Rolls razor. The opposite end of this blade is fitted with an inset steel ball. A setscrew with a knurled head is placed in the right end of the holder and is so arranged, that when it is tightened, its inner end engages the ball of the blade, thus securing it in an immovable position in the holder.

*To Insert Blade.*—The blade is placed in the depression of holder in such a manner that the two small pins on the left engage in proper depressions in blade. The setscrew is tightened, and the instrument is ready for use. Insertion and removal of blades are operations requiring but a few seconds to accomplish.

## CONCLUSIONS

A microtome blade suitable for all light routine work has been described. It has been found to function as well as any of the commonly used appliances and is superior from the standpoint of safety and the ease with which sharpening can be accomplished.

It is economical to purchase and maintain, and it offers no storage problem when not in use.

Appreciation is extended at this time to Mr. Raymond C. Corby, of Rolls Razors, Inc., for his aid and counsel during this investigation.

The apparatus described here is distributed by A. S. Aloe Co., St. Louis, Mo.

## A SIMPLE METHOD OF STAINING SPIROCHETES IN ROUTINE PARAFFIN SECTIONS\*

WITH REMARKS REGARDING DISTRIBUTION OF SPIROCHETES IN TISSUES

GABRIEL STEINER, M.D., DETROIT, MICH.

A METHOD of staining spirochetes in frozen sections was published in this JOURNAL December, 1937.<sup>1</sup> A modification of this technique for use with paraffin sections in routine procedure was sought, and a reliable method has been developed.

The procedure is as follows: the tissue is thoroughly fixed in 10 per cent formalin. Fresh tissue can be fixed in hot formalin (the formalin solution being changed once).

1. The tissue is dehydrated in graded alcohols, cleared in xylol, embedded in paraffin, cut at 9 to 10 micra, and mounted with albumin.

2. The mounted sections are deparaffinized in xylol and placed in absolute alcohol (two changes).

3. The sections are placed in a uranium nitrate-alcoholic gum mastic solution for one to one and one-half minutes. This time should not be exceeded, since a longer exposure to uranium nitrate interferes seriously with the subsequent staining of the spirochetes.

Uranium nitrate-alcoholic gum mastic solution:

Uranium nitrate (4%), alcoholic (absolute)	20 c.c.
Gum mastic (25%), alcoholic (absolute)	40 to 50 c.c.
Absolute alcohol	20 to 30 c.c.

This gives an 0.8 per cent uranium nitrate 10 to 12.5 per cent gum mastic solution in absolute alcohol. Two hundred cubic centimeters of the solution are necessary when using a glass tray carrying ten slides.

\*From the Department of Pathology, Wayne University College of Medicine, Detroit.  
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4. Wash the slides in at least three changes of distilled water. The first washing will cloud the water. A few streaks of the gum mastic may be shed in the second washing. If the third change of water is not clear, a fourth should be employed.

5. Place the slides in a 0.1 per cent aqueous silver nitrate solution for one to one and one-half hours. The staining dish containing the slides and silver nitrate is placed in a water bath at 100° C.

The solution of silver nitrate must be chemically pure. We have found the rectangular staining dish with the removable glass tray most convenient. A pan of tap water over an electric plate makes a satisfactory water bath. After the staining dish has been placed in the pan of warm tap water, the temperature is gradually raised to boiling.

6. Wash the slides in distilled water.

7. Dehydrate in ascending graded alcohols—80 per cent, 95 per cent, 100 per cent.

8. Place sections in 10 to 12.5 per cent alcoholic (absolute)-gum mastic solution for five minutes.

9. Repeat step 4.

10. Reduce in 5 per cent hydroquinone solution for twenty to thirty minutes. The staining dish containing the slides and hydroquinone solution is placed in a water bath at 100° C., the temperature having been raised gradually.

*Hydroquinone solution:*

Hydroquinone	10 gm.
Alcoholic gum mastic (12.5%)	1 c.c. (approximately)
Distilled water	200 c.c.

11. Wash thoroughly in distilled water, dehydrate in alcohol, clear in xylol, and mount in balsam.

12. If desired, the tissue may be counterstained with hematoxylin and eosin, cresyl violet, acid fuchsin, basic fuchsin, etc., after washing following reduction with the hydroquinone.

*Preparation of Stock Solutions:*

1. Uranium nitrate	4 gm.
Alcohol (100%)	100 c.c.

This solution may be kept indefinitely in an amber, glass-stoppered bottle.

2. Silver nitrate (C.P.)	1 gm.
Water (distilled)	1,000 c.c.

This is a stable solution if kept in an amber, glass-stoppered bottle.

3. Gum mastic powder	25 gm.
Alcohol (100%)	100 c.c.

Shake several times over a period of two days and then filter through a double filter. The filtrate is clear and has a yellow color.

The hydroquinone solution is unstable and must be prepared just prior to using.

The uranium nitrate-alcoholic gum mastic solution may be used not more than twice.

Separate staining dishes should be used for the uranium nitrate-gum mastic, for the aqueous silver nitrate, and for the hydroquinone solutions. Individual staining dishes should also be used for the distilled water and alcohol.

#### RESULTS

The color of the stained tissue depends upon the thickness of the section. Sections 8 to 9 micra in thickness have a deep yellow color; thicker sections tend to be brown, while thinner sections are pale yellow.

Occasionally reticular (argyrophil) fibers are stained in these preparations. However, because of their grayish color, as contrasted to the jet black of the spirochetes, reticular fibers are easily identified.

The time required for staining, if no counterstain is employed, is from two to two and one-half hours. Paraffin sections of primary and secondary syphilitic skin lesions, gummata, brain in general paresis, syphilitic aorta, the lung, liver, intestine and kidneys of congenital syphilitics, and organs of guinea pigs with *Leptospira* infection have been stained. The contrast between the glistening metallic black surface of the spirochetes and the indifferent yellow tissue is very marked. This marked contrast permits the identification of spirochetes when present in the tissue even in very small numbers.

*Advantages of This Method.*—1. Precipitates of metallic silver are prevented more easily than in other methods.

2. The method is faster than any other.

3. Only four special chemicals are required: (a) uranium nitrate, (b) silver nitrate, (c) gum mastic, and (d) hydroquinone.

Silver nitrate in 0.1 per cent solution is used; therefore, only small amounts of this rather expensive material are required.

4. This method fits into the usual routine technique used for paraffin sections in this country; therefore, alternating serial sections can be stained with hematoxylin eosin and for spirochetes. Counterstains are readily made on sections treated for presentation of spirochetes.

*Special Arrangements and Distribution of Spirochetes in Tissues.*—In organs of the congenital syphilitic with abundant spirochetes, an arrangement along the connective tissue bundles is very often found. The spirochetes show a tresslike accumulation on the connective tissue strands and between them. The longitudinal axis of the spirochetes runs parallel to the longitudinal direction of the fiber strands. In the outer parts of the walls of cross-sectioned blood vessels, dense masses of spirochetes are found arranged circularly. In epithelial structures (bronchial epithelium, intestinal epithelium) the spirochetes tend to be arranged radially to the lumen, parallel with the longitudinal axis of the columnar cells, and mostly between the cells. The basal membranes show a different arrangement insofar as here a circular distribution of spirochetes is seen. A clear-cut transverse section of a bronchus or an intestinal villus shows that the angle between the direction of the spirochetes in the epithelium and that of the organisms beneath the epithelium is usually

90 degrees (Fig. 1). In the muscularis of the intestinal tract the arrangement of spirochetes is in a line running parallel to the direction of the muscle fibers. Thus, in the layer of circularly arranged muscle fibers the spirochetes are arranged circularly also, and in the longitudinal muscular layer a longitudinal location of the spirochetes is found. In the leptomeninges the same rule applies—the spirochetes are arranged along the longitudinal course of the connective tissue, tangentially to the surface of the cortex of the brain.



Fig. 1.—Routine paraffin section, new stain, congenital syphilis. Spirochetes are arranged radially to the lumen of the duct parallel with the longitudinal axis of the columnar cells. In the basal membrane and the adjacent connective tissue spirochetes show a circular distribution. Note degenerating forms of spirochetes, rings, and knobs in parts of the duct. (X1,000.)

In parietic brains four different arrangements of spirochetes are discernible: first, a longitudinal arrangement in the adventitia of the veins, capillaries, and arteries along the course of these vessels running toward the depth of the gray matter (Fig. 2); second, an almost complete limitation to the gray substance of the central nervous system; third, an arrangement on the border line between the cortical gray matter and the white matter where the massed spirochetes tend to parallel the arrangement of the arcuate or U fibers;<sup>2</sup> and fourth, an arrangement around the larger nerve cells of the cortex, sometimes in dense accumulations<sup>3</sup> without penetrating into the nerve cells themselves. In parietic brains the complete absence of spirochetes in the vascular lumina is interesting to note, since in congenital syphilis the vascular spaces contain spirochetes. Previous examinations of a series of parietic brains with the method of frozen sections showed that, particularly in very acute and untreated cases, the parasites migrate from the leptomeninges into the cerebral cortex by way of the pial funnels of the vessels and the adventitial part of the vascular walls. A direct penetration from the leptomeninges through the superficial glial membrane and other structures of the outer part of the molecular layer was not seen. This finding is confirmed by the new method (Fig. 3). In the cerebellum of a parietic brain the same type of in-





Fig. 2.—Routine paraffin section, new stain. Early acute nontreated case of general paresis. Brain cortex. Longitudinal arrangement of spirochetes in the adventitial walls of cortical blood vessels. The spirochetes in the upper part of the figure are located in the wall of a capillary. ( $\times 950$ .)



Fig. 3.—Routine paraffin section, new stain. Early acute nontreated case of general paresis. Pia-arachnoid and pial funnel entering brain cortex. In the left lower part of the figure a semicircular shaped region of the brain surface, free from spirochetes, is seen. ( $\times 950$ .)

vasion was found. The spirochetes in the cerebellar substance itself are seen in parallel-running courses, and in the molecular layer sometimes they are arranged perpendicularly to the course of the blood vessels entering from the meninges.

A peculiar appearance of spirochetes should be mentioned, that is, their arrangement in large spherical or oval-shaped masses of closely packed individuals. This is seen in the acute and early stages of general paresis. These massive spirochetel accumulations are similar to those found in the liver and other organs of cases of congenital syphilis. Focal necroses in the tissues are produced by these dense masses, and this destruction is called "miliary necrosis," to be distinguished from miliary gummatous lesions. The histologic equivalent of these spirochetel massive coils is a miliary necrosis in the parietic brain also. These miliary necroses were first seen by Nissl<sup>4</sup> long before the disclosure of *T. pallidum* in the brain of paretics by Noguchi.<sup>5</sup> Later on, Hauptmann,<sup>6</sup> Schob<sup>7</sup> and others, could show that a definite and direct relationship exists between these massive spirochetel accumulations and the necrotic foci.

These massive balls of spirochetes in the acute and early stage of general paresis are found in the first, the second, and up to the fifth layer of the cerebral cortex, and on the borderline between gray and white matter. They are rarely found bordering the surface of the cerebral cortex adjoining the pia mater, but when this does occur, the shape of the spirochetel mass is semispherical or semioval, with the base on the cortical surface. On examination of the adjacent leptomeninges numerous spirochetes are also found, but the arrangement of these is in bundles of parallel-running spirochetes forming twisted threads.

One characteristic feature of the parenchymatously located massive spirochetel balls may be noted. The center shows a brownish color, whereas the spirochetes of the outer or peripheral parts are as black as usual.

Degenerating forms of spirochetes, spirochetel rings, disks and knobs of regular or irregular shape are stained black also, and are seen mostly on one end of the individual spirochete (Fig. 1). Occasionally a ring or knob is seen in the middle of a parasite.

#### COMMENT

The method herein described for staining spirochetes in paraffin sections is at least equal in its effects to other commonly used methods of staining spirochetes. The Levaditi,<sup>8</sup> Noguchi,<sup>5</sup> and Jahnke<sup>9</sup> methods are silver salt reduction methods which can be used only on tissue blocks. The Warthin-Starry<sup>10</sup> and Dieterle<sup>11</sup> methods may be useful for paraffin sections, but they are more complicated or take longer than this method.

The peculiar arrangements of *T. pallidum* in the tissues of syphilitics are not at present satisfactorily explained. Some of the difficulties of explanation depend on the fact that the spirochetes may survive the death of the host. After death of the infected host, the spirochetes may move and change their position. In animals (experimental syphilis of rabbits) after surgical removal

of syphilitic tissues and immediate fixation in formalin, spirochetal accumulation along the strands of connective tissue, and parallel-running with them can be seen. Similarly the adventitial and periadventitial accumulation of spirochetes is shown. Therefore, the characteristic distribution of spirochetes in the adventitial parts of the vascular walls must be accepted as an intravital phenomenon occurring in the body of the host.

The restricted location of the spirochetes which are limited to the gray matter and absent from the white matter of the brain in general paresis is in complete accord with the clinical and histologic findings. In general paresis inflammatory reactions are seen especially in the cortical gray matter and in the gray matter of the basal ganglia (nucleus caudatus, claustrum, putamen, and thalamus opticus). They consist of an infiltration of the adventitial sheaths of the vascular walls by plasma cells and lymphocytes. These are the same spaces in which in early stages of general paresis the spirochetes are carried.

#### SUMMARY

1. A new simple method of staining spirochetes in routine paraffin sections and its results are described.

2. A reason for the adventitial and perivascular round-cell infiltration seen in syphilitic tissue is suggested.

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# THE CONSTRUCTION OF AN INEXPENSIVE POWER WASHER AND THE DESIGNING OF ECONOMICAL AND EFFICIENT BRUSHES FOR THE CLEANING OF LABORATORY GLASSWARE\*

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THE problem of cleaning glassware for bacteriologic and serologic work becomes momentous if more than a few workers or students are to be supplied. Often there is not a great abundance of glassware, so the soiled glassware has to be cleaned and returned for use as rapidly as possible. Likewise the space provided for cleaning purposes usually does not provide accommodation for a staff of cleaners, nor do budgets make provisions for such a staff.

Since much of the glassware in bacteriologic laboratories is contaminated with pathogenic microorganisms, the material has to be sterilized before it can be handled by the workers. The sterilization is usually done by the quickest, most efficient and most economical method—autoclaving. Often the glassware contains blood, serum, Loeffler's medium, or such media as are used for growing the acid-fast group of microorganisms. After materials of this nature have been autoclaved, their removal from the glassware is usually very difficult. Often the autoclaving process makes traces of other material more difficult to remove. In order to clean such soiled glassware it must be thoroughly brushed, and, when cleaned by hand, this procedure requires much time and energy. Under these conditions it was our experience that the glassware was not satisfactorily cleaned. Mechanical washers available on the market were either too costly or impractical for the usual teaching and research laboratory, so we set about to construct an inexpensive power washer. We were handicapped by the lack of satisfactory brushes even when washing the glassware by hand.

*Power Washer.*—The articles used for constructing an inexpensive and practical power washer are:

- 1 General Electric motor, model 5KH43AB198,  $\frac{1}{8}$  HP, 110 V, AC, 60 cycle, single phase, cushion brace
- 1  $\frac{3}{4}$  inch chuck, tapered
- 1 shaft  $\frac{3}{4}$  inch diameter, 14 inches long, tapered to fit above chuck
- 2  $\frac{3}{4}$  inch flat boxes with grease cups
- 2 thrush bearings
- 2 collars for above thrush bearings
- 1 3 inch driver step pulley to fit  $\frac{3}{4}$  inch shaft
- 1 3 inch driver step pulley to fit  $\frac{1}{2}$  inch motor shaft
- 1 20 inch driver belt

\*From the Department of Bacteriology, School of Medicine, the University of Pennsylvania, Philadelphia.

Received for publication, March 9, 1939.

4 bolts for mounting shaft

4 bolts for mounting motor

2 three-way switches

1 male end for electric cord and a few feet of rubber-covered electrical extension cord

Miscellaneous pieces of lumber, nails, and screws for assembling cabinet.

The articles are all standard equipment and represent a total cost of less than \$22.00. The assembled unit is shown in Figs. 1 and 2.

At the base and on the inside of each of the two side panels is nailed a  $3\frac{1}{2}$  inch strip. Resting upon these strips and fastened to the sides is the base for mounting the motor. On the underside of this base is mounted the  $\frac{3}{4}$  inch shaft by means of the flat boxes. The motor is then mounted upon the base so as to give the proper tension on the belt. To the motor is attached an electrical extension cord by means of two three-way switches. A 1 inch hole is drilled in the front panel for the shaft, and the panel is secured in position by means of screws.

The purpose of a 3-way switch on each side of the washer is to enable the washer to be operated conveniently by either a left-handed or right-handed worker. The advantage of the tapered chuck is that the shaft may revolve in either direction without the danger of the chuck coming loose. The speed of the shaft should not be more than one-third that of the motor. Under ordinary conditions the motor runs at 1,725 r. p. m.; by means of the step-down pulleys the shaft revolves at 575 r. p. m. At this speed very little water is thrown from the brush, so we have not found it necessary to equip the washer with a shield to protect the worker. It requires only a few seconds to loosen the jaws of the chuck, insert a brush, and retighten the chuck. The compactness of the power washer enables it to be carried from one room to another or from one work table to another.

*Brushes.*—Satisfactory brushes, to be used by hand or with the power washer, for cleaning the usual laboratory glassware are not readily available on the market. The brushes which we now use for hand or machine work have been designed of a twisted-in-wire construction with the cooperation of the Fuller Brush Co., Industrial Division, Hartford, Conn., and have the following specifications (Fig. 3).

No. 1H706 for cleaning serologic tubes (10 mm. by 100 mm., outside diameter) by machine. Diameter  $\frac{1}{2}$  inch, brush length with tuft  $3\frac{1}{4}$  inches, overall length  $6\frac{3}{8}$  inches. Black bristle. Wire handle straight for inserting into chuck.

No. 1H707\* for cleaning serologic tubes by hand. Same specifications as for above brush 1H706, except that finger loop is left on end of handle so that the brush can be used by hand.

No. 1H708 for cleaning Wassermann tubes (13 mm. by 100 mm. outside diameter) by machine. Diameter  $\frac{1}{2}$  inch, brush length with tuft  $3\frac{1}{2}$  inches, overall length  $6\frac{1}{4}$  inches. Black bristle. Wire handle straight for inserting into chuck.

\*We have found it advantageous to have a few of the brushes made up with a loop on the end of the handle so that the brush can be used by hand. Occasionally it is desirable to brush a few tubes while the power washer is in use or a different sized brush is in the chuck.

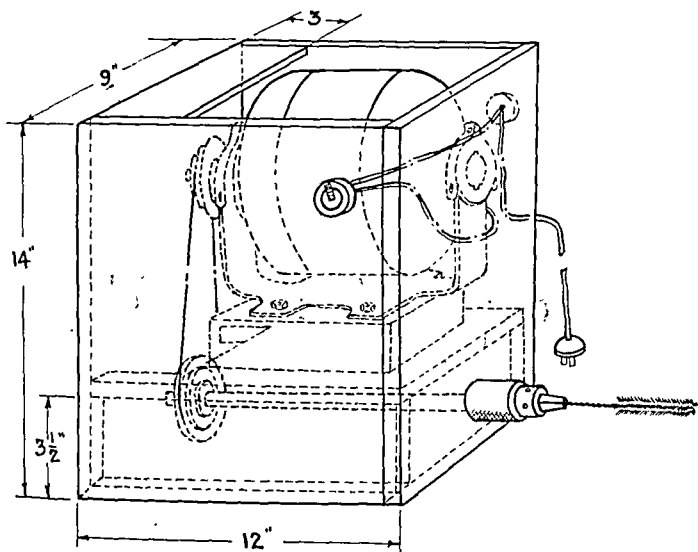


FIG. 1.—Electric test tube washer

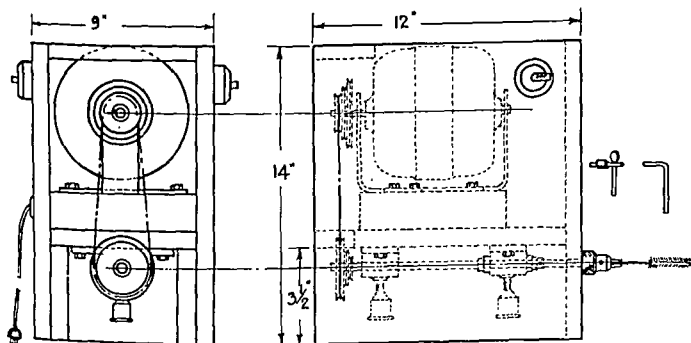


FIG. 2.—Electric test tube washer. A, Rear view; B, Side view, showing brush in place.

No. 1H709\* for cleaning Wassermann tubes by hand. Same specifications as for above brush 1H708, except that finger loop is left on end of handle so that the brush can be used by hand.

No. 1H704 for cleaning ordinary test tubes (16 mm. by 150 mm. outside diameter) by machine. Diameter  $\frac{5}{8}$  inch, brush length with tuft  $3\frac{1}{8}$  inches, overall length  $8\frac{1}{2}$  inches. Black bristle. Wire handle straight for inserting into chuck.

\*See footnote bottom page 212.

No. 1H705\* for cleaning ordinary test tubes by hand. Same specifications as for above brush 1H704, except that finger loop is left on end of handle so that the brush can be used by hand.

No. 1H703 for cleaning large test tubes (25 mm. by 200 mm. outside diameter) and 50 c.c. centrifuge bottles by machine. Diameter 1 inch, brush length with tuft  $3\frac{1}{4}$  inches, overall length  $10\frac{1}{2}$  inches. Black bristle. Wire handle straight for inserting into chuck.

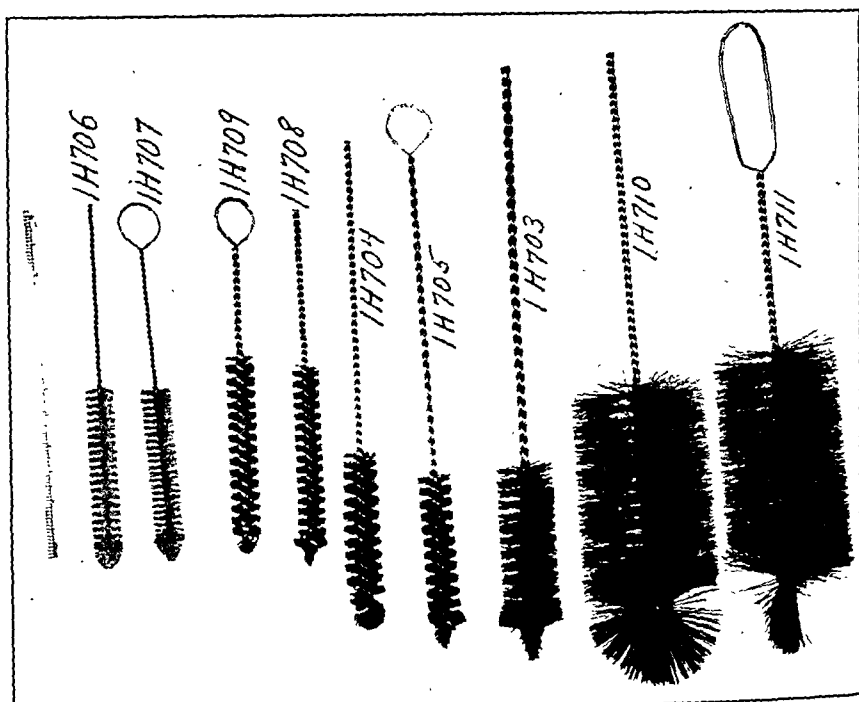


Fig. 3.—Brushes designed for machine or hand cleaning of laboratory glassware. For a description of the individual brushes see text.

No. 1H710 for cleaning 250 c.c. centrifuge bottles and 125 c.c. Erlenmeyer flasks by machine. Diameter  $2\frac{3}{8}$  inches, brush length with tuft 5 inches, overall length 11 inches. Black bristle. Wire handle straight for inserting into chuck.

No. 1H711\* for cleaning 250 c.c. centrifuge bottles and 125 c.c. Erlenmeyer flasks by hand. Same specifications as for above brush 1H710, except that loop is left on end of handle so that the brush can be used by hand.

If it is necessary to clean glassware other than the above-mentioned standard sizes, satisfactory brushes can be designed as were these brushes.

*The Cleaning Process.*—Contaminated glassware is autoclaved before handling, the cotton stopper removed, and any fluid poured out while still warm. The cotton stoppers are removed from other glassware, and any liquid, if present, is poured out. The glassware is then completely submerged in dish pans of suitable size (we have found the oblong, white enamel baby baths most convenient), containing about half an ounce of Char-Lab washing powder† dis-

\*See footnote bottom page 212.

†Obtainable from Elmer and Amend, New York, N. Y.

solved in about 3 gallons of warm water. The solution of washing powder is brought to a boil and boiled for about fifteen minutes. The solution is cooled or replaced with cool tap water until the glassware is cool enough to handle.

A brush of the proper size is placed in the chuck of the power washer, the chuck is tightened, and the brush set in motion by turning on the power washer. It is best for the cleaner to wear rubber gloves and also to wear over the rubber glove of the right hand an ordinary white canvas glove which serves two very good purposes: (1) as a wash cloth and (2) as added protection. A handful of tubes is picked up out of the wash water with the left hand, and without emptying the wash water out of the tubes, they are cleaned one by one on the revolving brush. A tube is grasped firmly by the right hand and shoved over the revolving brush as far as possible. There is practically no danger whatsoever of the end of the brush breaking the tube as the brushes are constructed with a very good tuft. By the time the tube has been shoved over the brush until the tuft comes in contact with the end of the tube, the inside of the tube is cleaned, and the tube may be withdrawn from the brush.

Usually the outside of the tubes is also soiled, as with wax pencil marks. These wax marks are very easily removed, if while the tube is still on the brush, the grip on the tube is released slightly so as to allow the tube to revolve against the canvas glove which is being worn over the rubber glove.

It is the practice of our cleaner to run the tube over the brush as far as possible, release the grip on the tube slightly so as to allow the tube to revolve against the gloved hand, hold the palm of the gloved hand against the end of the tube for a second, then run the gloved hand along the sides of the tube to the open end of the tube, tighten the grip on the tube, pull the tube off the brush and drop it into a vat of running warm tap water. The glassware after being well rinsed in the running warm tap water is given several rinsings in two vats of clean, distilled water.

Under no consideration should the hand be removed from a tube while it is revolving on the brush! The power washer should be stopped by turning off the switch, and the hand removed from the tube only after the tube has stopped revolving.

It has been our experience that the breakage of glassware with the power washer is less than when the glassware was cleaned by hand with the usual test tube brushes. By actual timing, glassware can be cleaned in one-fourth the time which was required for brushing by hand. The glassware is cleaned better, and is not fogged by the numerous repetitions of the cleaning process. Another economical feature is that the glassware does not have to be further cleaned by sulfuric acid-potassium dichromate solution.

#### SUMMARY

An inexpensive power washer is described which is far superior to the hand process for cleaning glassware.

Brushes for cleaning the usual laboratory glassware are described which are more economical and efficient than any heretofore obtained.

These innovations bring about great savings in time and materials.



# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**COCCIDIOIDAL GRANULOMA, Arrested Pulmonary, Cox, A. J., and Smith, C. E. Arch. Path. 27: 717, 1939.**

Four cases of completely arrested coccidioidal granuloma of the lungs or of the bronchial lymph nodes in man are reported. In none of these were the granulomatous lesions a factor in the cause of death.

Similar arrested lesions could be produced at will by injecting small numbers of spherules of a virulent strain of the fungus *coccidioides* intravenously into white rats or guinea pigs. The minimum fatal inoculum for rats inoculated in this way was approximately 10,000 spherules.

In nearly all the arrested experimental lesions organisms remained viable for periods up to two and a half years. In a human case organisms were cultured fifteen years after they had been first demonstrated.

It is concluded that instances of arrested coccidioidal granuloma are more frequent than has heretofore been realized, that they probably outnumber the fatal cases of this disease, and that they are not restricted to the region of the San Joaquin Valley, Calif. The relationship of arrested coccidioidal granuloma to the benign clinical disease known as "Valley fever" is as yet unknown. Although the lesions present a histologic picture of inactivity, they may contain viable fungi for many years and constitute a possible source of dissemination.

**BLOOD: Total Differential and Absolute Leukocyte Counts and Sedimentation Rates Determined for Healthy Persons Nineteen Years and Over, Osgood, E. E., Brownless, I. E., Osgood, M. W., Ellis, D. M., and Cohen, W. Arch. Int. Med. 64: 105, 1939.**

The proportion of lymphocytes is higher and the proportion of neutrophile lobocytes (polymorphonuclears) is lower than the textbook figures. The probable explanation for the retention of erroneous figures in the textbooks is that these figures represent fairly closely the usual counts of patients sick enough to be attending an outpatient clinic or to be hospitalized, but who are not suffering from a generally recognized cause of marked leucocytosis. The dangers of using such persons as normal subjects are obvious.

In the age group 19 years and over, the leucocyte count is lower, the neutrophile percentage is higher, and the lymphocyte percentage is lower than the corresponding figures for younger persons. It is of interest that the absolute number of neutrophile lobocytes (polymorphonuclears) averages 4,000 per c.mm. for all ages from 4 to 30 years, except for the age group 8 to 14 years.

The sedimentation rates form a skew curve, with the greatest number of determinations falling in the lower ranges. It is probable that the rate of 15 mm. in forty-five minutes, which includes 80 per cent of the results, represents the strict upper limit of normal values, and that the higher rates are due to mild chronic infection in the tonsils, teeth, or sinuses not detectable in the routine physical examination.

There are no significant age or sex differences in the total, differential, or absolute leucocyte counts, or in the sedimentation rates in persons 19 to 30 years of age.

**TISSUE: Differential Stain for Cell Types in the Pancreatic Islets, Gomori, G. Am. J. Path. 15: 497, 1939.**

**Fixation:** The thickness of pieces of tissue should not exceed 2 mm. in order to ensure rapid and complete penetration by the fixative. Aqueous fixatives, such as formalin, Bouin's, Zenker's, and Stieve's solution, or Zenker-formalin, may be used. The best fixative proved to be a modified Bouin's solution in which half the amount of acetic acid is replaced by sulfosalicylic acid (formalin 1 part, saturated picric acid solution 4 to 5 parts, acetic and sulfosalicylic acid 2.5 per cent each). For use, dilute this fixative with equal parts of distilled water. The staining of tissues fixed in any fluid can sometimes be greatly improved by refixing the sections before staining in the undiluted solution for twelve to twenty-four hours. The removal of mercury salts from tissues is done by the routine iodine method.

**Embedding:** Embed tissues in paraffin.

**Oxidation:** Deparaffinized (and refixed, if necessary) sections are treated for one minute with a solution containing about 0.3 per cent each of potassium permanganate and of sulfuric acid. The sections are rinsed in water and then decolorized with a 1 to 5 per cent solution of potassium metabisulfite. After decolorization the sections are thoroughly washed in water. Without oxidation the beta granules will not take the stain. For sections of pituitary, oxidation, though it will enhance the color contrast, is not strictly necessary.

**Staining:** Stain in a well-ripened solution of chromium hematoxylin for from fifteen minutes to one hour under microscopic control. The beta granules should be a deep blue, and the cytoplasm of the alpha cells should be unstained. In the pituitary the basophiles should be a deep blue, and the oxyphiles unstained. Any mucoid material is stained a deep blue. The staining solution is made up as follows:

Mix equal parts of a 1 per cent aqueous solution of hematoxylin and of a 5 per cent solution of chromium alum (potassium chromium sulfate). The brownish mixture is ripened by the addition of about 3.5 c.c. of a 5 per cent potassium dichromate solution plus a few drops of sulfuric acid to each 100 c.c. of the mixture. The process of ripening will take one or two days. The solution is ready for use as soon as it is a deep blue black with a purplish tinge. It should be filtered before use as a precipitate will form repeatedly on its surface. For some time the staining power and selectivity of the solution increase, but as the solution becomes older, the staining time has to be prolonged. Solutions that do not stain deeply enough in thirty-five to forty minutes are better discarded as they often do not stain with sufficient precision.

After staining in this chromium hematoxylin solution, rinse the sections in water and transfer to alcohol containing 1 per cent of hydrochloric acid. In this acid-alcohol solution the color of the section becomes a clearer blue. Rinse again and counterstain rather heavily with any of the routine red or orange acid dyes. Phloxine and ponceau de xylinine have been found to be especially suitable. The latter is used in a 0.5 per cent solution with 1 per cent of acetic acid. Rinse in water and differentiate in a 5 per cent solution of phosphotungstic acid until the acid dye is completely removed from the connective tissue. Only the strongly oxyphilic structures, such as erythrocytes, muscle fibers, oxyphiles, and alpha cells, remain stained. Carry sections through graded alcohols, clear in xylol, and mount in balsam.

**TRICHINELLA SPIRALIS: Incidence of Infection in Man, Dogs and Cats in the New Orleans Area, Sawitz, W. Arch. Path. 28: 11, 1939.**

Examination of human diaphragms and pectoral muscles obtained in 400 routine unselected necropsies disclosed 24 cases of infection with *T. spiralis* in the New Orleans area, an incidence of 6 per cent. The compressor method detected 2 cases; the digestion method, 24. Of the 23 cases in which both the diaphragm and the pectoral muscle were available, the diaphragm was found infected in 20 cases, or 87 per cent, and the pectoral muscle in 13 cases, or 56.5 per cent. Surveys in which diaphragms only were examined would thus miss 13 per cent of the cases. The average number of larvae of *T. spiralis* found in the diaphragm was 0.35 per gm.; the average number in the pectoral muscle, 0.22 per gm. The diaphragm is,

therefore, not only qualitatively but also quantitatively the better tissue for examination. No history of clinical symptoms of trichinosis was found in any of the 24 cases. With increasing age the incidence of trichinella infection increased. The highest incidence was found in Negro females (6.66 per cent); the lowest, in white females (5.8 per cent).

The incidence of trichinella infections in 300 dogs in the New Orleans area was found to be 1.3 per cent; the incidence in 90 cats was found to be 10 per cent. The incidence in cats is considered to serve as an indicator of the endemicity of trichinella infection in an area.

**TUBERCULIN TEST, Evaluation of the Patch, Vollmer, H., and Goldberger, E. W.** *Am. J. Dis. Child.* 57: 1272, 1939.

A comparative study of the tuberculin patch test (Lederle-Vollmer) and the Mantoux test has been carried out on 678 children.

Among 417 children admitted to the wards of the Mount Sinai Hospital, the reliability of the tuberculin patch test, as compared with the Mantoux test performed with 0.1 mg. of old tuberculin, was 100 per cent.

Two hundred and sixty-one tuberculous children of the Sea View Hospital showed 100 per cent conformity between the tuberculin patch test and the Mantoux test with first strength solution of purified protein derivative, or 0.01 to 1 mg. of old tuberculin. Only one child with positive anergy failed to react to the patch test as well as to the Mantoux test with first strength purified protein derivative. This child reacted positively to the Mantoux test with second strength purified protein derivative.

Consequently, it seems that the Mantoux test with 0.1 mg. of old tuberculin or less, or with first strength solution of purified protein derivative, can safely be replaced by the tuberculin patch test. The routine follows:

1. The tuberculin patch test (Lederle) is applied and read two days after removal of the patches.
2. Negative reactors to the patch test are retested with the Mantoux test with 1 mg. (0.1 c.c. of solution 1:100) of old tuberculin or second strength solution (0.005 mg.) of purified protein derivative.
3. Since higher concentrations of tuberculin occasionally cause pseudopositive reactions, both tests are repeated if there is any discrepancy. If the discrepancy remains, the Mantoux reaction is regarded as the deciding one.

This procedure is advocated for exact diagnosis in hospital wards. For general use in practice and for mass examinations the tuberculin patch test alone may suffice.

**SULFANILAMIDE, Cyanosis From Use of, Bigler, J. A., and Werner, M.** *Am. J. Dis. Child.* 57: 1338, 1939.

From the available accumulated data it is evident that the cause and the mechanism of production of the cyanosis that so frequently occurs during sulfanilamide therapy are still not clear. While in some instances spectroscopic examination of the blood has revealed the presence of sulphemoglobin and methemoglobin bands, in other instances it has given negative results. The cyanosis may be present in the presence or absence of nonfunctional hemoglobin. When nonfunctional hemoglobin is present, it has some, but not all, of the characteristics of methemoglobin. Further, the cyanosis does not seem to be dependent on the oxygen saturation of the arterial blood. Certainly, further studies of this problem are necessary for clarification.

**THYROID GLAND, Histologic and Histochemical Structure of the Normal, Hertzler, A. E.** *Arch. Surg.* 38: 417, 1939.

A study of the thyroid glands of supposedly normal persons presents confusing variations. Many show changes parallel with those found in goiters from patients with goiter heart, that is, cardiac disease which disappears after removal of the goiter. Naturally these studies of the nongoitrous gland lead surgeons to ask themselves certain questions. What is the meaning of these changes in chemical reaction of the colloid in allegedly normal thyroids? No one knows, but it is known that these changes are found in goiters

from patients with "goiter hearts" and that in such a patient the heart recovers when the goiter is completely removed. So much is incontrovertible. This well-demonstrated clinical fact causes one to consider that possibly a degenerated thyroid gland, even though it has not acquired goitrous dimensions, may injure the heart. If this is true, the presence of a "goiter heart" demands the removal of the thyroid gland whether it is goitrous or not. First of all, one must recognize a "goiter heart" by clinical examination. It has been demonstrated to the author's satisfaction that, in the absence of a palpable goiter, if "goiter heart" is present, the removal of the thyroid, enlarged or not, rids the patient of the cardiac disturbance. These observations must be multiplied a thousandfold.

This presents the original question: How big must a thyroid gland be in order to become cardiotoxic? The corollary is, what changes in the thyroid gland must be present before it can be declared the cause of the cardiac symptoms? This is easily decided if one follows the aftercourse. If the patient recovers from the cardiac signs, one may conclude that the part removed was the cause. Multiply such an observation by thousands, and the conclusion is as accurate as one can hope to achieve. The patient, collectively speaking, is always right. If anatomic study does not corroborate clinical experience, one must extend the laboratory study. The surgeon should remember that when he sends a goiter to the laboratory, he does so to secure, not a diagnosis, but an opinion.

This is the problem before surgeons today. Since it is now known that total removal of the thyroid gland from the adult is a matter of indifference to the patient, it becomes merely a question of operative technique.

The tinctorial, as well as the histologic, study of the thyroid gland indicates the presence of certain changes which occur with advancing years. These changes parallel to some extent those with "goiter heart."

The physicians' knowledge of the relation of the nongoitrous gland to diseases of the heart is at present represented by a large question mark, but even this may presage an advance in understanding of the relationship between the nongoitrous and the goitrous gland.

The similarity of structure suggests that the effect of the thyroid gland is a matter of function, not of size. One may determine the nature of this function by removing the gland and observing the results.

**ANEMIA, Exogenous Pernicious, Alsted, G.** *Am. J. M. Sc.* 197: 741, 1939.

A case of pernicious anemia is reported in a man of 43. It is shown that the development of anemia was due to deficiency of "extrinsic factor," continuous during seven to eight years. Treatment with "extrinsic factor" exclusively was followed by complete recovery and complete restoration of acid secretion in the stomach. The patient was controlled during seven months subsequent to the discontinuation of the specific treatment, and no relapse was observed.

The case is conceived as a case of exogenous pernicious anemia.

**SYPHILITIC REAGIN, in Blood and Spinal Fluid, Wiener, A. S., and Derby, I. M.** *Arch. Dermat. & Syph.* 39: 999, 1939.

The concentrations of syphilitic reagin in serum and spinal fluid were compared in a series of 1,245 pairs of specimens by a quantitative flocculation technique. It was found that, with the method used, titers as high as 500 were attained in the blood serum, whereas the titer of the spinal fluid never exceeded 16. Moreover, the amount of reagin in any spinal fluid was practically always less than that of the corresponding serum. The bearing of these observations on the question of the site of origin of syphilitic reagin in the spinal fluid of neurosyphilitic patients is discussed.

**SULFANILAMIDE, Observations Concerning the Absorption of, From the Large Intestine in Man, Turell, R., Marine, A. W. M., and Nerb, L.** *Brooklyn Hosp. J.* 1: 90, 1939.

From the experiments reported, it is evident that in man sulfanilamide administered by rectum in aqueous solution is absorbed from the large intestine and from the isolated rectum.

No changes were observed in the normal mucous membranes of the rectum and sigmoid following the introduction of sulfanilamide solution into the rectum.

It has been shown that the concentration of sulfanilamide in the blood, after administration of the drug by rectum, is sufficiently high to recommend this route of administration when sulfanilamide cannot be given by mouth.

**"WINDOW PATCH" TEST, Guild, B. T. Arch. Dermat. & Syph. 39: 807, 1939.**

A description is given of a technique of patch testing which the author calls the "window" patch test. It is valuable for the following reasons:

1. It makes possible the frequent applications of buffered solutions to the test site and thus may be used to study the influence of alkalinity or acidity on the development of reactions of sensitivity or irritability of the skin.

2. It is useful for testing reactions of the skin to volatile substances, as repeated applications may be made beneath the glass window.

3. It provides constant visibility of the test site for both the patient and the physician; consequently, the development of serious reactions may be avoided.

4. The test site may be exposed to light; therefore, the method should be valuable in studying certain dermatoses caused by substances, the cutaneous reactions to which may depend on exposure to light.

The method follows: A microscope slide is cut with a glass cutter into three equal sections, each 1 inch (2.5 cm.) square, and the edges are well smoothed by means of an emery wheel or a suitable file. A strip of adhesive plaster,  $\frac{3}{4}$  inch (1.9 cm.) wide and about  $2\frac{1}{2}$  inches (6.2 cm.) long, is applied to each of three sides of the glass square, so as to secure it to the skin and at the same time allow a portion of the glass to remain uncovered for the purpose of observation. The substance to be used in the test is then placed directly on the skin and covered by the central clear portion of the glass square. The ends and free edges of the strips of adhesive tape are then stuck tightly to the skin. The device is commonly referred to in my laboratory as the "open" patch, because the upper edge of the glass has been left free, making it possible to insert fluids beneath the glass.

**PNEUMONIA, VIRUS of Infants Secondary to Epidemic Infections, Goodpasture, E. W., Auerbach, S. H., Swanson, H. S., and Cotter, E. F. Am. J. Dis. Child. 57: 997, 1939.**

Five cases of a hitherto undescribed virus infection of the lungs of infants, especially following measles and whooping cough, have been observed and recorded.

The virus invasion usually appeared to be secondary and tended to pave the way for bacterial infection of the lungs.

The presence of the virus was indicated by the occurrence of nuclear inclusions in epithelial cells of the trachea and bronchi and their mucous glands, and of the alveolar epithelium. Rapid necrosis of the affected cells occurred, resulting in ulceration of surfaces.

The virus appeared to be different from that of herpes simplex and from the agent of the so-called inclusion disease of infants.

Experimental inoculation of infected lung tissue into rabbits, mice, opossums, chicken embryos, and a *Macacus rhesus* monkey, failed to establish the infection.

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

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### Rural Medicine\*

AMONG the more popular "indoor sports" of the moment appears to be the preparation of data—statistical or otherwise—concerned with availability or nonavailability of medical care; it is somewhat unfortunate that some, at least, of these bear evidence of having sprung from bias or of having been conceived from preformed opinion.

Alive to this problem and thoroughly familiar with rural environment, the staff of the Mary Imogene Bassett Hospital at Cooperstown, New York, decided to analyze their own experience and, for this purpose, called a conference to consider the results obtained.

The facts elicited are presented in this volume and are discussed by outstanding workers interested in this phase of medical practice.

The volume is divided into four main sections representing the subjects discussed at the four meetings: Rural Morbidity; Health Department Programs and School Health Programs in Rural Areas; Postgraduate Medical Education in Rural Areas; and Economics of Rural Medicine.

A bibliography of rural medicine concludes the book.

This is perhaps one of the more important of the recent contributions to this field, if not, indeed, among the most important, principally, perhaps, because it sets out to *prove* nothing. The object was to *find out* the true state of affairs in a representative rural community and from this, if possible, to draw such inferences and conclusions as were legitimate. The particular value of this study lies, first, in its *factual* character and, second, in its broad approach.

It is a volume which deserves careful reading for its stimulating effect and might well be studied by both the radical and the conservative in the ranks of the medical profession. The sociologist, economist, and all who are busy in this matter may well read and study it with profit.

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### Crystalline Enzymes†

IN THIS monograph the author presents the results of a series of investigations on the proteolytic enzymes and upon the isolation and chemistry of bacteriophage. While the latter has not yet been crystallized nor, indeed, yet shown to be definitely an enzyme, the results of experiments with it are similar to those obtained with enzymes and so presented with them. Six chapters are given to the discussion of enzymes and the results obtained with them in crystallized form; the seventh chapter discusses bacteriophage. An appendix gives the methods used in full detail. There is a full bibliography and an index.

This book should be of great interest to the biochemist and physiologist.

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### Advances in the Therapeutics of Antimony‡

WITH the use of antimony taking on renewed importance in the treatment of a variety of diseases of man and beast, this most excellent résumé of the present status of the pharmacology and therapeutics of antimony compounds should be most welcome. As a

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\*Rural Medicine. Proceedings of the Conference Held at Cooperstown, New York. Cloth, 258 pages, \$3.50. Charles C. Thomas, Springfield, Ill.

†Crystalline Enzymes, the Chemistry of Pepsin, Trypsin and Bacteriophage. By John H. Northrop, Member of Rockefeller Institute for Medical Research. Cloth, 176 pages, 48 figures, \$3.00. Columbia University Press, New York.

‡Advances in the Therapeutics of Antimony. By Hans Schmidt, Ph.D., and F. M. Peter, M.D. With a Preface by Philip Manson-Bahr, M.D. Paper, 257 pages, 10 diagrams. Georg Thieme, Leipzig.

preface Dr. Philip Manson-Bahr has written most interestingly of the history of antimony. The authors then discuss the clinical experience with antimony compounds in human and veterinary medicine, citing in a very attractive and novel manner the world literature. The synthesis of various preparations is discussed with proper bibliographic references. Then there are some thirty pages devoted to tests in which antimony preparations were used on animals infected experimentally. The authors close with a thorough description of the pharmacology of antimony and with a discussion of some of the newer preparations available.

The reviewer feels that this is a book that should be read by all those interested in the therapeutic application of antimony to infectious diseases and those interested in chemotherapy. It is an excellent book.—*H. B. Haag.*

### Manual of Urology\*

THE avowed purpose of this book is to present "the fundamentals of urology in such a way that they can be easily grasped by the student approaching this subject."

This second edition, extensively revised and rewritten, will without doubt maintain the reputation achieved by its predecessor, characterized as "the most compact and yet basically complete book on urology available."

Professor Le Compte, in addition to being master of his subject, possesses the happy knack of being able to present it in a clear-cut and assimilable manner.

A good book for the physician as well as the student.

### Textbook of Medicine†

THIS, the fourth edition, of a well-known English reference text, has been thoroughly revised to include the advances made since the appearance of the preceding edition in 1936.

Though necessarily in covering the whole field of medicine, the articles are of various lengths and in the main brief, they are nevertheless comprehensive and cover the subjects systematically as well as concisely.

A good "refresher" reference text.

### Pye's Surgical Handicraft‡

AS THE editor of this book says in his preface, when a medical book, and especially a book concerned with so flexible a subject as surgery, retains its place for fifty-four years, it can be safely assumed that it is a good book, a useful book, and one which may be relied upon.

The eleventh edition of this standard work worthily carries on the reputation and tradition of its predecessors.

The house surgeon and surgical intern, indeed the intern at large, will find this volume of inestimable value. The physician at large may well and profitably add it to his shelf of working references.

Altogether it is an excellent book, and in an emergency is well worth many times its price.

\*Manual of Urology. By R. M. Le Compte, M.D., F.A.C.S., Professor of Urology, Georgetown University Medical Department. Cloth, ed. 2, 295 pages, 54 figures, \$4.00. Williams & Wilkins Co., Baltimore, Md.

†Textbook of Medicine. Edited by J. J. Conybeare, M.C., and D. M. Oxon, F.R.C.P., Physician to Guy's Hospital, London. Cloth, ed. 4, 1112 pages, 26 plates, 24 figures. Williams & Wilkins Co., Baltimore, Md.

‡Pye's Surgical Handicraft. A Manual of Surgical Operations. Minor Surgery, and Other Matters Concerned with the Work of the House Surgeon. Edited by Hamilton Bailey, F.R.C.S., Surgeon Royal Northern Hospital, Essex County Council; External Examiner in Surgery, University of London. Cloth, ed. 11, 512 pages, 375 illustrations, \$6.00. Williams & Wilkins Co., Baltimore, Md.

### Peripheral Vascular Disease\*

THE increasing popularity of the monograph is a logical aftermath of the constant and varied changes in the understanding both of disease in general and of diseases in particular. For, devoted to a single subject, the monograph is free to discuss it comprehensively and even exhaustingly.

In this book Collens and Wilensky have made a notable contribution to the study of peripheral vascular disease, a subject of great interest and increasing importance, not only to the specialist but also to the practitioner at large by whom the vast majority of such cases are first seen.

The book has two main parts: the first, in the authors' words, "devoted to anatomy, pathological physiology, symptoms, signs, methods of examination, diagnosis and differential diagnosis of vascular disorders"; the second, "a description, summary and evaluation of every known method of treatment, the application of the basic laws of physiology to treatment, the indications for the methods of treating each individual disease and the eventual prognosis." These objectives are fully attained in the book.

This book is not a theoretical, abstract, or superscientific discussion, but a clear, sane, and usable presentation based not only upon a thorough familiarity with the pertinent literature but also upon a comprehensive practical clinical experience. The book is well illustrated.

It is a book useful to the specialist, but perhaps even more valuable to the physician in general, and as such can be highly commended.

### The Harvey Lectures†

THE thirty-fourth of a series of lectures, delivered under the auspices of the Harvey Society of New York, contains the following addresses: Some Aspects of the Intermediary Metabolism of the Steroid Hormones (Guy F. Marrian); The Significance of the Albumin Fraction of the Serum (Ashley Weech); Heat Loss From the Human Body (Eugene F. DuBois); Proteins as Chemical Substances and as Biological Components (E. J. Cohn); Observations on the Pathology of Rickets (Edwards A. Park); Distribution of Enzymes in Tissue and Cells (K. Linderström-Lang); Genic and Hormonal Factors in Some Biological Processes (C. H. Danforth); and Biological Oxidation and Vitamins (Albert Szent-Gyorgyi).

The authors are all of international repute, and their lectures represent the outstanding advances in their respective fields.

### Sclerosing Therapy‡

MUCH of the discussion of sclerosing therapy has arisen from failure to appreciate its limitations and contraindications as well as from attempts to utilize it without a thorough comprehension of its difficulties and niceties of technique.

In this book four of the outstanding exponents of this method discuss its applications, each in the particular field in which he has had extensive experience.

*The Injection Treatment of Hernia* is fully expounded by A. F. Bratrud, assistant professor of Clinical Surgery and Director of Clinic for Ambulant Treatment of Hernia, University of Minnesota. George F. Hock, Associate Attending Urologist, St. Luke's Hos-

\*Peripheral Vascular Disease: Diagnosis and Treatment. By William S. Collens, B.S., M.D., Metabolist, Chief of Clinic for Peripheral Vascular Disease, Chief of Diabetic Clinic, Israel Zion Hospital, Brooklyn; and Nathan R. Wilensky, M.D., Assistant in Clinic for Peripheral Vascular Disease, Assistant in Diabetic Clinic, Israel Zion Hospital. Cloth, 243 pages, 77 illustrations, 3 color plates, \$4.50. Charles C. Thomas, Springfield, Ill.

†The Harvey Lectures, 1938-1939. Series XXXIV. Cloth, 279 pages, illustrated. \$1.00. Williams & Wilkins Co., Baltimore, Md.

‡Sclerosing Therapy. The Treatment of Varicose Veins, Hemorrhoids, Hernia and Hydrocele by Injection. Edited by Frank C. Yeomans, M.D., F.A.C.S., Professor of Proctology and Attending Surgeon, New York Polyclinic Medical School and Hospital. Cloth, 336 pages, 185 illustrations, \$6.00. Williams & Wilkins Co., Baltimore, Md.



pital, New York, discusses *The Injection Treatment of Hydrocele*, while *The Injection Treatment of Varicose Veins* is covered by Harold J. Shelley, Assistant Surgeon, St. Luke's Hospital, New York, and *The Injection Treatment of Hemorrhoids* by F. C. Yeomans.

This book is exceptionally practical in its aspect and detailed and precise in its presentation. It is an excellent book for those interested in this subject.

### You Can't Eat That\*

A GENERAL cook book for those with allergy. Part I describes allergy and the allergic type of reaction. The historical review of the early experimental work leading up to present-day clinical allergy is very well done, somewhat in the de Kruifian manner.

Part III, by far the largest section, contains recipes. These are not merely the wheat, egg, and milk avoidance recipes so familiar to those dealing with allergic persons. Instead, they cover the field of cookery for allergies in a comprehensive manner, stressing the need for tasty and palatable dishes to replace others which are usually so much regretted by those who must avoid them.

The third section contains general information on foods. It has Tuft's list of ingredients of nationally advertised food preparations, to which many additions have been made.

This volume should be of real service to those who must practice allergic dietary avoidances and who are fortunate enough to have someone in the kitchen willing to go to the trouble of trying new things.

### Recent Advances in Physiology†

THIS, the sixth edition of a justly popular review series, has been thoroughly revised to bring the text in line with the advances in this field. Among the entirely new additions are discussions of the physiology of bone, the Carrel-Lindbergh perfusion apparatus, problems of carbohydrate metabolism, and cortical control of muscular movements. A useful book for those desiring a succinct but thorough review.

### Recent Advances in Hematology‡

SO extensively revised as to be practically rewritten, with four additional color plates and sixteen added text illustrations, this new edition of Piney will, without doubt, maintain and enhance the repute achieved by its predecessors.

An excellent, comprehensive, and authoritative review of an important subject.

\*You Can't Eat That. A manual and recipe book for those who suffer either actually or mildly (and perhaps unconsciously) from food allergy. By Helen Morgan. Foreword by Dr. Walter C. Alvarez, of the Mayo Clinic. Harcourt, Brace and Co., New York, 1939.

†Recent Advances in Physiology. By C. Lovatt Evans. Revised by W. H. Newton, M.D., M.Sc., Reader in Physiology, University of London, University College. Cloth, ed. 6, 490 pages, 109 illustrations, \$5.00. P. Blakiston's Son & Co., Philadelphia, Pa.

‡Recent Advances in Hematology. A. Piney, M.D., Ch.B., M.R.C.P., Assistant Physician, St. Mary's Hospital for Women and Children, London. Cloth, ed. 4, 312 pages, 34 illustrations, 8 color plates, \$5.00. P. Blakiston's Son & Co., Inc., Philadelphia, Pa.

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## *CLINICAL AND EXPERIMENTAL*

### CHANGES IN THE CHEMISTRY OF CEREBROSPINAL FLUID DURING ENCEPHALOGRAPHY\*

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THE widespread use of encephalography and ventriculography in the diagnosis and prognosis of pathologic conditions of the central nervous system has stimulated the study of the effects of withdrawal of large amounts of cerebrospinal fluid. It has been found that there is usually a pleocytosis in the cerebrospinal fluid subsequent to encephalography<sup>1,2</sup> and that the sugar content is usually increased toward the end of the procedure. There has been some disagreement as to whether or not the protein content of the cerebrospinal fluid is affected by the displacement of fluid by air. According to the recent work of Schwab and von Storch,<sup>2</sup> the protein content decreases slightly during the course of the procedure and returns to normal toward the end.

In examining the literature on the subject of the cerebrospinal fluid reaction to encephalography, it becomes apparent that only a very few constituents of the cerebrospinal fluid have been studied to any extent, viz., cells, protein, and sugar. Furthermore, most investigations have been carried out after encephalography, while very little work has been done on the changes which occur in the cerebrospinal fluid during the course of the procedure. It appeared worthwhile, therefore, to study the changes, if any, of a number of chemical constituents of the cerebrospinal fluid during the course of encephalography. Accordingly, we have studied the effect of displacement of cerebrospinal fluid by air on the sugar, chloride, nonprotein nitrogen, total protein, calcium, and phos-

\*From the Children's Hospital of the Cook County Hospital, the Sarah Morris Hospital for Children of the Michael Reese Hospital, and the Department of Biochemistry, Nelson Morris Institute for Medical Research, Chicago.

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TABLE I  
ENCEPHALOGRAPHY

CASE	DIAGNOSIS	AMOUNT OF AIR IN- JECTED (C.C.)	CELL COUNT	PHOS- PHA- TASE (UNITS)	TRYP- SIN (UNITS)	ANTI- TRYP- SIN (UNITS)	LIPASE (UNITS)	TRI- BUTY- RINASE (UNITS)	ESTER- ASE (UNITS)	AMY- LASE	MG. PER 100 C.C. OF FLUID				
											SUGAR	CHLO- RIDES	TOTAL PROTEIN	CAL- CIUM	PHOS- PHORUS
L. G.	Epilepsy	0	0	1.3	0	0	0	0	0	0.5	58	738	15	5.6	1.9
		25		1.1	0	0	0	0	0	0.5	60	726	14	5.1	1.9
		45		1.2	0	0	0	0	0	0.5	61	721	14	5.1	1.9
		60		2.0	0	0	0	0	0	0.5	63	721	14	5.3	1.9
		80		2.5	0	0	0	0	0	0.5	63	727	18	5.2	1.9
M. R.	Cerebral agensis with mild hydrocephalus	0	1	1.1	0	7	0.4	1.3	0	0	53	724	17	5.4	2.1
		40	--	2.8	0	0	0.3	1.9	0	0	55	718	19	5.5	2.2
		60	--	3.5	--	--	0.5	0.8	0	--	57	711	25	--	2.2
		80	6	3.8	0	0	0.5	1.0	0	0	63	711	27	5.5	2.2
M. G.	Cerebral agensis	0		0.8	0	0	1.8	1.1	0	2.0	46	722	22	5.4	1.2
		20		0.9	0	0	0.6	1.9	0	--	49	714	23	--	1.1
		40		2.7	0	0	0.9	0.2	0	2.0	47	727	24	4.8	1.5
		65		5.7	0	0	2.1	1.8	0	1.1	51	709	30	5.7	1.5
A. L.	Cerebral agensis	0	3	0.4	0	0	0	0	0	0	44	732	13	5.3	2.0
		20	--	1.3	0	0	--	--	--	0.6	47	732	13	--	1.6
		40	--	1.8	--	--	0	0.4	0	--	48	728	15	5.1	1.8
		65	9	2.2	0	0	0	0.4	0	0.5	52	714	19	5.3	1.8
P. S.	Cerebral agensis with optic atrophy	0		0.8	0	0	0.9	1.2	0	1.4	51	718	14	4.6	1.8
		20		1.2	--	--	0.2	1.5	0	--	--	---	18	4.5	2.2
		70		3.0	0	0	1.0	1.0	0	1.0	53	717	20	4.6	2.0
M. C.	Cerebral agensis	0		5.2	0	0	0	0	0	0	52	717	23	5.7	1.5
		40		1.7	0	0	0	0	0	0	59	700	19	5.5	1.5
		60		1.8	0	0	0	0	0	0	58	716	23	5.9	1.6
H. K.	Hemihypertrophy of the body	0	3	0.6	0	0	1.2	0	0	1.0	56	713	11	4.0	0.8
		20	--	0.3	0	0	1.3	0	0	0.4	60	715	10	4.5	0.8
		60		3.2	0	0	0	2.5	0	0.4	77	706	25	4.7	0.8

TABLE I—CONT'D  
 ENCEPHALOGRAPHY

CASE	DIAGNOSIS	AMOUNT OF AIR IN- JECTED (C.C.)	CELL COUNT	PHOS- PHA- TASE (UNITS)	TRYP- SIN (UNITS)	ANTI- TRYP- SIN (UNITS)	LIPASE (UNITS)	THI- BUTY- RINASE (UNITS)	ESTER- ASE (UNITS)	AMY- LASE	SUGAR	CHLO- RIDES	TOTAL PROTEIN	CAL- CIUM	PHOS- PHORUS
MG. PER 100 C.C. OF FLUID															
R. P.	Epilepsy	0		0	0	0	0.4	0	0	2.0	58	713	14	4.5	1.4
		40		1.7	0	0	0.5	0.7	0	--	77	715	20	--	1.3
		60		2.2	0	0	0.4	0.7	0	1.0	56	706	31	5.3	1.4
E. B.	Epilepsy	0	1	0.4	0	0	0	0	0	0.8	54	730	19	5.0	1.3
		90	5	1.5	0	0	0	0	0	0.8	60	729	13	4.9	1.5
M. M.	Mental deficiency	0	3	0.2	0	5	0.3	0	0	0	47	718	13	5.5	1.0
		40		0.3	0	12	0.4	0	0	0	51	723	13	5.8	1.1
D. D.	Microcephalus	0	3	0.7	0	10	0.5	0.4	0	0.4	65	723	18	6.0	1.1
		40	1	12.0	0	0	0.5	9.4	0	0.8	--	--	--	--	1.6
		70		0.9	0	0	0.4	0.4	0	1.0	54	720	21	5.9	1.6

phorus contents of the cerebrospinal fluid. Since it has been found<sup>3</sup> that the enzymatic properties of the cerebrospinal fluid vary in different pathologic conditions of the central nervous system, studies were also made of the trypsin, phosphatase, lipase, tributyrinase, esterase, amylase, and antitrypsin of the cerebrospinal fluid. The results of these studies are reported in this paper in the hope that they will add to the present knowledge of encephalography and offer a broader basis for the interpretation of the effects of that procedure.

#### METHODS

Specimens of cerebrospinal fluid were obtained from children in whom encephalography was indicated for diagnostic or prognostic purposes. The encephalographies were done by the open method, namely, the removal of small amounts of cerebrospinal fluid and the introduction of a lesser amount of air by means of a syringe.<sup>4</sup> Avertin was used as an anesthetic. In all cases the fluid was withdrawn after an overnight fast, and the studies of the chemical constituents and of the enzymes were started within one or two hours. The fluids included in this study were clear and colorless to begin with and remained so until the end. Occasionally, the last portion of the fluid became slightly turbid, most likely due to the admixture of carbon dioxide from the injected air. We did not include in this study any fluid that contained blood either grossly or microscopically.

Sugar was determined by the method of Somogyi;<sup>5</sup> chlorides by the method of Van Slyke;<sup>6</sup> total protein by the method of Ayer, Dailey, and Fremont-Smith;<sup>7</sup> calcium by the Clark and Collip modification of the Kramer-Tisdall method;<sup>8</sup> phosphorus by the method of Bodansky<sup>9</sup> and nonprotein nitrogen by the method of Koch and McMeekin.<sup>10</sup>

Phosphatase was determined by the method of Bodansky<sup>11</sup> except that 2 c.c. of fluid were used instead of 1 c.c. Trypsin and antitrypsin were determined by a slight modification of the method of Anson and Mirsky;<sup>12</sup> lipase, tributyrinase, and esterase by the method of Crandall and Cherry;<sup>13</sup> amylase by a modification of the method of Ross and Shaw.<sup>14</sup> The details of the methods for the determination of enzymatic activity, as well as a description of the units employed, are given elsewhere.<sup>3</sup>

#### RESULTS

We shall consider first eleven encephalographies in which determinations were made of the enzymatic activity of the fluids as well as of the simpler chemical constituents. The various constituents will be taken up separately. The results are tabulated in Table I.

*Sugar.*—In 9 of the 11 cases the sugar content increased during the course of the encephalography. In one case it decreased, and in one case no significant change<sup>\*</sup> was observed. The average sugar content of the first portion of fluid was 53 mg. per cent; that of the last portion studied was 61 mg. per cent.

\*By "significant change" is meant a change of  
3 mg. per cent in the case of sugar, total protein, and nonprotein nitrogen;  
10 mg. per cent in the case of chlorides;  
0.3 mg. per cent in the case of calcium;  
0.2 mg. per cent in the case of phosphorus.

*Total Protein.*—In 7 of the 11 cases the total protein content of the fluid increased as cerebrospinal fluid was replaced by air. In one case it decreased, and in 3 instances there was no significant change. The average protein content of the fluid obtained before injection of air was 16 mg. per cent; that of the fluid obtained after the injection of the maximum amount of air was 22 mg. per cent.

*Chlorides.*—There was no marked variation in the chloride content of the cerebrospinal fluid during encephalography. In 4 instances the chloride value decreased slightly; in 7 there was no significant change. The average value of the chloride content was 722 mg. per cent before injection of air; after injection of air it was 716 mg. per cent.

*Calcium.*—In 3 instances the calcium was higher in the last portion than in the first. In the other 8 there was no significant change. The average value of the first portion was 5.1 mg. per cent; that of the last portion was 5.4 mg. per cent.

*Phosphorus.*—In 4 instances the phosphorus content of the fluid increased during the course of the encephalography. In 6 cases no significant changes were noted, and in one case the phosphorus value decreased. The average value of the first portion of fluid was 1.46 mg. per cent; that of the last portion was 1.58 mg. per cent.

*Cell Count.*—The cellular content of the last portion of fluid was greater than that of the first portion.

*Phosphatase.*—In 9 cases the phosphatase activity of the final portion of fluid was markedly higher than that of the fluid obtained before injection of air. In 5 of these cases the phosphatase activity increased progressively as the amount of injected air increased. In 2 instances the phosphatase activity decreased slightly after injection of 20 c.c. of air, and then increased when 60 c.c. of air had been injected. In one instance the activity rose sharply after injection of 40 c.c. of air, then decreased after injection of another 20 c.c. In only one instance was the phosphatase activity of the first portion of fluid greater than that of the subsequent portions. In this case (M. C.) the activity of the first portion of fluid was considerably higher than that normally found.<sup>3</sup>

*Trypsin and Esterase.*—No traces of these enzymes were found. This was to be expected in view of the fact that the fluids of this group were "normal" in the sense that the cellular and chemical constituents were normal. We have shown elsewhere<sup>3</sup> that such fluids rarely, if ever, have trypsin or esterase activity.

*Lipase.*—Lipase was either absent entirely, or present in small traces in 8 of the 11 cases studied. In 2 cases (M. C. and P. S.) lipolytic activity was present in the first portion of fluid, decreased in the intermediate portions, and returned to the original value in the last portion. Finally, in one instance (H. K.) lipolytic activity was present in the first and second portions of fluid, but not in the last portion. In 10 instances, therefore, there were no significant differences in the lipolytic activity of the first and last portions of fluids; in one case a decrease was found.



*Tributyrylase.*—Tributyrylase was either absent entirely, or present in small traces in 7 of the 11 cases studied. In 3 instances tributyrinase was present in all the portions of fluid studied. In 2 of these there was no significant difference in activity between the first and last portions; in the other case the last portion had greater activity than the first. In one case (H. K.) the early portions of fluid had no activity, while the final portion had a high tributyrinase value. In 10 instances, therefore, there was no significant difference between the first and last portions; in one case the activity of the last portion was greater than that of the first portion.

*Amylase.*—Amylase was absent completely, or present in small traces in 5 cases; in 2 other cases amylase was present, but not changed during the course of the encephalography. In 3 instances the amylolytic activity of the last portion of fluid was less than that of the first portion, in one case it was greater than that of the first portion. The changes, however, were small.

*Antitrypsin.*—Antitrypsin was completely absent in 8 cases. In 2 instances small amounts were present in the first portion of fluid, but not in the later portions. In one case a slight increase was noted in the last portion of fluid. On the whole, the observed changes were not significant.

The results obtained with the 11 fluids of this group can be summed up as follows: The sugar, total protein, and cellular contents of the fluids generally increased during the course of encephalography. The same was true of the phosphatase activity. Occasional increases were found in the calcium and phosphorus contents of the fluids after displacement of cerebrospinal fluid by air. The chlorides decreased slightly in a few cases. No significant changes were observed in the other constituents studied.

In a severe case of hydrocephalus simultaneous lumbar and ventricular punctures were done. Ventricular fluid was then replaced by air, and studies were made of the portions of fluid removed after injection of 150, 250, and 350 c.c. of air.

There was no appreciable difference in the sugar contents of the five portions of fluid. Chlorides were higher in the lumbar fluid than in the ventricular fluid removed before introduction of air. However, after the injection of air, the chlorides rose. Total protein was also higher in the lumbar than in the ventricular fluid. After 250 c.c. of air had been injected, the protein content of the fluid rose, only to fall again on further injection of air. The calcium values of lumbar and ventricular were practically equal. After injection of 150 c.c. of air the calcium value fell, then rose to the original level after 350 c.c. of air had been injected. The phosphorus content of the ventricular fluid was slightly higher than that of the lumbar fluid and was not affected by the displacement of fluid by air (Table II).

Phosphatase activity was higher in the lumbar fluid than in the ventricular. It was not affected by injection of air. Trypsin and esterase were not found in any of the fluid portions. A small amount of antitrypsin was present (6 units). In the last portion of fluid, obtained after 350 c.c. of air had been injected, the antitryptic power was 17 units. Lipase was present



TABLE III  
SUGAR, CHLORIDES, NONPROTEIN NITROGEN, TOTAL NITROGEN, AND CALCIUM IN ENCEPHALOGRAPHY FLUID

CASE	DIAGNOSIS	AMOUNT OF AIR INJECTED (c.c.)	SUGAR		CHLORIDES		NONPROTEIN NITROGEN		TOTAL PROTEIN		CALCIUM	
			BEFORE AIR (MG.)	AFTER AIR (MG.)	BEFORE AIR (MG.)	AFTER AIR (MG.)	BEFORE AIR (MG.)	AFTER AIR (MG.)	BEFORE AIR (MG.)	AFTER AIR (MG.)	BEFORE AIR (MG.)	AFTER AIR (MG.)
S. S.	Mental deficiency	60	43	65	710	719	18	17	28	45	4.3	4.8
J. M.	Mental deficiency	100	63	69	723	709	16	19	37	18	4.4	4.3
M. B.	Mental deficiency	70	--	--	723	718	14	14	35	51	5.0	5.4
N. J.	Cerebral agenesis	60	50	60	702	695	10	14	26	21	5.0	5.3
E. S.	Epilepsy	95	61	68	714	717	14	15	15	28	3.9	3.8
M. S.	Epilepsy	75	46	48	707	719	14	14				
J. B.	Epilepsy	80	47	54	756	765	25	31				
P. B.	Epilepsy	120	49	54	638	671	18	18				
G. B.	Epilepsy	90	64	87	685	730	--	--				
M. S.	Epilepsy	135	43	47	684	702	17	17				
S. A.	Epilepsy	100	61	57	720	717	16	16				
M. K.	Little's disease	105	54	78	724	718	13	14				
A. S.	Little's disease	130	50	51	722	723	20	21				
J. M.	Little's disease	130	39	76	711	711	19	19				
S. K.	Little's disease	80	49	47	744	742	28	31				
B. J. S.	Postencephalitic syndrome	80	50	86	749	737	17	21				
S. R.	Postencephalitic syndrome	70	31	40	726	728	14	14				
L. S.	Postencephalitic syndrome	100	47	56	725	725	17	15				
V. W.	Mental deficiency	80	46	58	715	691	12	14				
E. K.	Mental deficiency	80	37	59	737	724	19	19				
B. P.	Mental deficiency	90	59	65	713	716	21	17				
F. A.	Hydrocephalus	200	51	74	707	707	20	20				
W. A.	Microcephalus	60	45	46	691	665	17	17				
R. V. H.	Chronic encephalopathy	160	42	82	723	716	14	14				
D. S.	Lead encephalopathy	170	30	50	737	728	16	16				
Average values			49	61	716	716	17	18	28	33	4.5	4.7

in varying amounts in all portions of the fluid, but the small quantitative changes observed were without significance. Tributyrinase activity, unlike phosphatase, was greater in the ventricular fluid than in the lumbar fluid. It did not change significantly on injection of air. Amylase was higher in the portion of ventricular fluid obtained before injection of air than in the lumbar fluid. It decreased somewhat on injection of air.

In general, in this ventriculography the greatest contrasts were between the lumbar fluid and the portion of ventricular fluid obtained before the injection of air. The chemical composition of the ventricular fluid was practically unaffected by the injection of air.

We consider next the results obtained in a series of encephalographies in which the effect of displacement of cerebrospinal fluid by air on the sugar, chloride, nonprotein nitrogen, total protein, and calcium contents of the fluid were studied (Table III).

TABLE IV  
SUMMARY OF RESULTS

CONSTITUENTS	NO. OF CASES STUDIED	NO. OF CASES INCREASE WAS NOTED AFTER INJECTION OF AIR	NO. OF CASES DECREASE WAS NOTED AFTER INJECTION OF AIR	NO. OF CASES NO SIGNIFICANT CHANGE WAS NOTED	AVVERAGE VALUE BEFORE INJECTION OF AIR	AVERAGE VALUE AFTER INJECTION OF AIR
					MG. PER CENT	
Sugar	35	28	2	5	50	61
Chlorides	36	4	10	22	717	716
Nonprotein nitrogen	24	5	1	18	17	18
Total protein	16	10	3	3	20	26
Calcium	16	5	0	11	4.9	5.2
Phosphorus	11	4	1	6	1.42	1.55
Phosphatase	11	10	1	0	----	----
Trypsin	11	0	0	11	----	----
Antitrypsin	11	1	2	8	----	----
Lipase	11	0	1	10	----	----
Tributyrinase	11	1	0	10	----	----
Esterase	11	0	0	11	----	----
Amylase	11	1	3	7	----	----

*Sugar.*—In 19 of the 24 cases studied the sugar content of the fluid was increased after injection of air. In 4 cases there was no significant change; in one case it decreased. The average value before injection of air was 49 mg. per cent; after injection of air, it was 61 mg. per cent.

*Chlorides.*—In 4 out of 25 cases studied, the chlorides decreased; in 4 cases they were increased, and in 17 cases there was no significant change. As a whole, neither the decrease nor the increase was very significant. The average value before injection of air was 716 mg. per cent; after injection of air it was still the same.

*Nonprotein Nitrogen.*—Of 24 cases in which the nonprotein nitrogen content was determined before and after the injection of air, no significant change was found in 18. In one instance the value decreased; in 5 it increased. The average value before injection of air was 17 mg.; after injection it was 18 mg. per cent.

*Total Protein.*—In the 5 cases studied for the protein content, the total protein was increased in 3 and decreased in 2. The average before injection of air was 28; after injection of air it was 28 mg. per cent.

*Calcium.*—In 3 cases the calcium content increased slightly on injection of air; in the other 2, no significant change was noted. The average calcium values of the first and last portions of fluid were 4.5 and 4.7 mg. per cent, respectively.

*Cells.*—The cellular content of the last portion of fluid was greater than that of the first portion.

The results of the entire study are summarized in Table IV. In general, sugar, total protein, and phosphatase, as well as cellular content, rose during the course of encephalography. Calcium and phosphorus showed a slight tendency to rise. Nonprotein nitrogen, chlorides, antitrypsin, trypsin, lipase, tributyrinase, amylase, and esterase did not change significantly when cerebrospinal fluid was displaced by air.

#### DISCUSSION

What is the cause of the changes observed? There are two possible explanations for the rise in the sugar content of the fluid. One is in terms of the difference in location of the fluid making up the various portions withdrawn during encephalography. The first portion of fluid removed is lumbar fluid. Since it is known that the sugar content of cisternal fluid is slightly greater<sup>15</sup> and that of the ventricular fluid considerably greater<sup>16</sup> than that of lumbar fluid, it follows that the higher sugar content of fluid obtained after injection of air may be due in part to its being ventricular fluid. However, this explanation is not satisfactory for the other chemical changes observed during encephalography. Ordinary cisternal or ventricular fluid is generally lower in cellular and protein contents than lumbar fluid.<sup>16, 17</sup> This is not the case in encephalography where the latter portions of fluid contain more cells and protein than the first portion. A second explanation for the increase in sugar is in terms of the hyperglycemia which has been found in many cases of encephalography.<sup>18-20</sup> Such hyperglycemia, either alone or in conjunction with the increased meningeal permeability associated with an aseptic meningeal reaction,<sup>21</sup> would tend to increase the cerebrospinal fluid sugar.

The increase in cells and protein may also be due to an aseptic meningeal reaction caused by the presence of air in the ventricles and subarachnoid space. This hypothesis is supported by the fact that in aseptic meningitis induced experimentally by intraspinal insufflation of air,<sup>22</sup> a marked pleocytosis results, together with an increase in total protein. On the other hand, Riser and Planques<sup>23</sup> have found increased cellular and protein contents during the course of continued simple spinal drainage. This seems to indicate that the chemical changes in the cerebrospinal fluid during encephalography are due in part to the presence of air and in part to the readjustment of the cerebrospinal system to loss of fluids.

The importance of an aseptic meningeal reaction is further indicated by the tendency of calcium to rise during encephalography. In view of the very low

calcium content of brain tissue<sup>24</sup> and the increased calcium content of the cerebrospinal fluid often found in meningitis,<sup>25</sup> the most likely source of calcium increase during encephalography is increased meningeal permeability. In those instances where the calcium values rose considerably during the procedure the total protein content also rose sharply (see Table I: R. P., H. K.; Table III: M. B., S. S.). The combination of increased calcium and total protein may be an indication of meningeal or chorioidal irritation. In some cases, calcium, sugar, and total protein were hardly affected during encephalography. This may point to a very slight meningeal reaction. However, in a few cases the sugar content remained practically constant while a marked cellular reaction took place (see Table III: A. S., M. S., W. A., and S. A.). Unfortunately, studies of the calcium and total protein contents were not made in these instances. The subject of an aseptic meningeal reaction in encephalography requires further investigation, although at present it seems to be the simplest hypothesis which can be offered for the chemical changes in the cerebrospinal fluid during encephalography.

Probably the most interesting finding reported in the present paper is the phosphatase activity of the fluid, which seemed to parallel the amount of cerebrospinal fluid removed. Since the blood has considerable phosphatase activity, it seems possible that some phosphatase may enter the cerebrospinal fluid because of the increased meningeal permeability caused by irritation of the meninges and chorioid plexus. It is difficult to explain, however, why other enzymes, such as lipase, tributyrinase, and amylase, as well as antitrypsin, would not also enter the cerebrospinal fluid. It would be necessary to hypothesize different barrier permeabilities for the different enzymes. There are at present no adequate experimental data on which to base such an hypothesis. We have found<sup>26</sup> that in infectious meningitis some enzymes probably do cross the meningeal barrier, but no quantitative relationships have been found between the degree of involvement of the meninges and the degree of permeability of the meninges to enzymes.

Another possibility is that phosphatase from brain tissue reaches the subarachnoid space via the perivascular spaces in the course of the adjustment of the cerebrospinal fluid to the presence of air.

The importance of effects, such as those discussed above, is perhaps more strongly indicated by recent work on simple and forced drainage of cerebrospinal fluid. Kubie and Retan<sup>27-32</sup> have studied at great length the physiologic and clinical effects of spinal drainage coincident with the intravenous injection of hypotonic saline. They often found an increase in the cell count of the fluid during forced drainage, and are of the opinion that this is due to drainage of the perivascular channels. Retan has used "forced perivascular drainage" in the treatment of diseases of the central nervous system, such as meningitis, encephalitis, poliomyelitis, syphilis, and chorea. Fay<sup>33</sup> has suggested that the beneficial results obtained by Kubie and Retan were due to the increased blood volume of the brain caused by removal of cerebrospinal fluid, rather than to perivascular drainage. He considered that encephalography produces a forced drainage in that all the cerebrospinal fluid may be readjusted when an appro-

priate amount of air has been introduced to take its place. This possibility is supported by the work of Riser and Planques,<sup>23</sup> who found, in an experimental study of simple and forced drainage in dogs, that the number of cells and the protein content of the fluid increased without change in the chlorides during the course of drainage. Histologic examination of the brain and spinal cord of the dogs showed a mild lymphocytosis in the arachnoid meshes, but no active cellular proliferation in the perivascular spaces of the brain and spinal cord. Riser and Planques concluded that the increases in lymphocytes and protein are merely reactions of the chorioid plexus and meningeal vessels, and do not indicate drainage of the neural parenchyma itself.

Since the changes observed during encephalography, simple drainage, and forced drainage are similar with respect to those constituents of the cerebrospinal fluid which have been studied, it seems reasonable to assume that the three processes have some factor in common. This factor may be the adjustment of the cerebrospinal fluid to changes in intracranial hydrodynamics caused by the removal of cerebrospinal fluid. In encephalography the adjustment may be complicated by an aseptic meningeal reaction due to the presence of air. In forced drainage the intravenous injection of hypotonic saline may be a complicating factor, but this is not borne out by the chemical studies which have so far been made. Further experimental studies on forced drainage, emphasizing possible changes in the enzymatic activity, calcium, and phosphorus, may aid in the solution of this problem.

#### CONCLUSIONS

1. The chemical constituents of the cerebrospinal fluid were studied during the course of encephalography. The constituents investigated were sugar, chlorides, nonprotein nitrogen, total protein, calcium, phosphorus, phosphatase, lipase, tributyrinase, esterase, amylase, and antitrypsin.

2. The phosphatase, sugar, and total protein contents of the cerebrospinal fluid are increased after injection of air into the spine and ventricles.

3. Calcium and phosphorus increased in some cases, but less generally than phosphatase, sugar, and total protein.

4. Chlorides, nonprotein nitrogen, lipase, tributyrinase, esterase, amylase, and antitrypsin underwent no consistent significant changes.

5. The changes observed are most probably due to an aseptic meningeal reaction caused by the presence of air in the ventricles and subarachnoid spaces. Similar changes, however, have been reported during simple and forced drainage of cerebrospinal fluid.

6. The increase in sugar content may also be due in part to the fact that the later portions of encephalography fluid are ventricular rather than lumbar or cisternal.

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## HEAT LABILE FACTORS NECESSARY FOR THE PROPER GROWTH AND DEVELOPMENT OF CATS\*

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THIS report covers a three-year study of the effect of raw and cooked foods upon cats. The mother cats were placed on special foods four to six months before breeding. The diet of cats given raw food consisted of raw meat and dry feed raw milk; the diet of cats given cooked food consisted of cooked meat and dry feed raw milk. Other studies have shown that the properties of dry feed raw milk are similar to those of pasteurized milk. Both groups were given a liberal amount of cod-liver oil to insure an adequate supply of vitamins A and D. The study deals chiefly with the offspring produced from these mother cats.

All of the cats used in this experiment were kept in large outdoor pens. The sides and tops of the pens were screened with chicken wire only, so that the cats had the benefit of all the sunshine available. Each pen was divided into two portions—a yard 4 feet wide and 12 feet long, with a 7 foot ceiling, and a roofed section 6 feet square with a 6 foot ceiling, a wooden floor and bedding. The floor of the pen was bare earth covered with fine sand.

During this three-year period, 63 living kittens were born of parents fed raw food, with an average weight of 119 gm. at birth, and 47 kittens were born of parents fed cooked food, with an average weight of 100 gm. There were 4 dead kittens born in the raw-food group and 16 in the cooked-food group. An example of the effect of the diet of the mother upon the birth weight of the offspring is the following: Cat H. F. fed a cooked-food diet produced a litter of 4 kittens, whose average weight was 77 gm. She was then placed on a raw-food diet, and her litter for the next year consisted of 5 kittens, with an average birth weight of 116 gm., while the following year (still on a raw-food diet) the litter consisted of 3 kittens, with an average weight of 137 gm. The opposite result may be produced by taking a mother cat who has been on raw food and placing her on cooked food.

Cat D given a cooked-food diet produced a litter of 5 kittens, with an average weight of 105 gm., and the following year her litter consisted of 6 kittens, with an average weight of 91 gm.

If the mother cat is kept on cooked food for more than two years, she usually dies during delivery.

The cats fed cooked food occasionally produce a premature or full-term litter of stillborn kittens. In several instances mother cats on a cooked-food diet died during delivery. One cat was unable to deliver her kittens even though in labor for seventy-two hours. Delivery complications such as these have not been found in the case of cats placed on a raw-food diet.

\*From the Research Department, Pottenger Sanatorium, Monrovia.  
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The cats fed cooked food were smaller in build; though the bones of their legs were smaller in diameter, they were frequently longer and their fur development was much poorer than that of the cats fed raw foods. Muscle tone and visceral development of cats fed raw meat was much greater than that of cats fed cooked meat. Disturbance in the genital development and descent of the testes was common in male kittens of mother cats on cooked food. Cats on cooked-food diet were of a much more nervous disposition than cats on raw-food diet; they paced the pens, they were not very friendly, and they were often quite vicious.

One of the first defects noticed in the cats fed cooked food was poor dental development. The temporary teeth of both the cats fed cooked foods and those fed raw foods seemed well developed. However, when the permanent incisors displaced the temporary ones, the cats on the cooked-food diet usually developed three or four irregularly spaced, uneven, crowded incisors instead of the usual six. This was true of both the upper and lower jaws. Although a few of the cats on the raw-food diet had this same condition, it occurred from three to four times as often in the cats on the cooked-food diet as in those on raw food. This condition became even more pronounced in the second and third generation of cats on the cooked-food regime.

The femurs of these animals were removed, and the entire bone analyzed for its calcium and phosphorus content. The kittens of both the mother cats who had been fed raw food and those who had been fed cooked food had approximately the same amount of calcium and phosphorus at birth; quite often this was within 2 or 3 per cent of that found in the femur of the mother cat. After the first two weeks a marked depletion of the calcium and phosphorus of the bone occurred. This no doubt corresponds with the period of greatest growth. Two months later the bones of the kittens on the raw-food ration were gradually approaching normal in respect to these salts while those of the kittens on the cooked-meat ration still lagged behind. This effect was still more pronounced in the second and third generations; that is, the bones of second and third generation kittens given cooked food, even after they reached adult life, were markedly deficient in calcium and phosphorus content. Corresponding litters of kittens fed raw food had from two to three times as much calcium and phosphorus in their bones. If the first, second, and third generations were kept on a cooked-food diet, the third generation kittens were in such poor condition that they lived at the longest only two months. We were unable to carry our experiments beyond the third generation on the cooked-food diet, because no cats survived beyond that time.

Our experience has been that once a deficiency is produced in kittens it cannot be reversed even under intense therapy. A well-developed cat can be maintained in a healthy state on deficient diets if thyroid and adrenal hormones are added to the dietary. A deficient kitten, however, even if given raw food, thyroid and adrenal hormones, insofar as we have seen, does not become a normal cat.

If a mother cat has been on a cooked-food diet from twelve to eighteen months, it takes at least three years on a raw-food diet before her kittens begin to approach normal.



Upon the death of the cats a complete post-mortem examination was made. A study of microscopic sections of the lungs of second and third generation cats showed that cooked food was responsible for the production of certain respiratory conditions. The lungs of these cats showed hyperemia, some edema, and partial atelectasis, while those most deficient showed bronchitis and pneumonitis. The kittens fed raw food had normal lungs, although occasionally a cat was found whose lungs showed a mild hyperemia.

Five of the cats given cooked food showed suppurative inflammation of the thigh. Smears of the pus showed the presence of streptococci and staphylococci. The femurs were fragile and showed marked osteoporosis, with marked thinning of the cortex. In 2 of these 5 cats the bone had been partially eaten away and only chips and fragments remained.

In several cases we found a hypothyroid condition among the cats fed cooked food. The thyroids showed scanty colloid, and the acini were small. This was not observed in the thyroids of cats given raw food.

The ovaries of the cats fed cooked food showed, as a rule, only a few developing follicles and primordial ova, while those cats fed raw food showed active oogenesis with normal and large numbers of developing follicles. In males the testes of the cats fed cooked food showed a lessened number of spermatozoa and many of them showed an absence of spermatozoa but with active spermatogonia. Cats fed raw food showed active spermatogenesis.

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## STUDIES IN BLOOD PRESERVATION\*

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### FATE OF CELLULAR ELEMENTS IN RELATION TO POTASSIUM DIFFUSION

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IN A PREVIOUS publication<sup>1</sup> we reported a tenfold rise in plasma potassium of preserved blood. This was not due to bacterial contamination. It was increased by trauma, such as shaking, and was definitely modified by the area of the interface between the sedimented cells and the overlying plasma.

The question was raised as to whether this increase in plasma potassium was a pure diffusion process or whether it was due to actual cell destruction. This aspect of the problem is reported here.

### METHOD

Five cubic centimeters of freely flowing venous blood were collected in each of 35 sterile round-bottomed test tubes containing 5.0 mg. of heparin as an

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anticoagulant. After inverting each tube three times, they were plugged with cotton and kept in a refrigerator at approximately 4° C. throughout the period of the experiment.

Each day one tube was taken from the refrigerator, and centrifuged for one hour. After 0.5 c.c. of plasma had been removed for potassium analysis,<sup>2</sup> and after the blood had been thoroughly mixed by inverting the tube fifteen times, the following determinations were made:

- (1) Red cell count.
- (2) Hemoglobin (Hellige).
- (3) White cell count.
- (4) Differential white cell count.
- (5) Platelet count.
- (6) Mean cell diameter of red blood cells (Halometer method).

### RESULTS

The donor, A. J., was a professional man of International Group 0.

	VENOUS BLOOD		
	AT START OF PHLEBOTOMY	AT END OF PHLEBOT- OMY (590 C.C.)	
Hematocrit	46.0	44.7	per cent cells
Plasma specific gravity	1.0286	1.0266	
Plasma proteins	7.38	6.70	gm. per cent
Whole blood potassium	212.0		mg. per cent
Plasma potassium	21.5		mg. per cent
Cell potassium (calculated)	434.0		mg. per cent
pH		7.51	

### VALUES ON INITIAL 5.0 C.C. SAMPLE\*

Red blood cell count	5,500,000.0	
Hemoglobin	14.2	gm. per cent
White blood count	7,000.0	
Differential white cell count		
Polymorphonuclear leucocytes	52.0	per cent
Eosinophilic leucocytes	2.0	per cent
Basophilic leucocytes	1.0	per cent
Lymphocytes	42.0	per cent
Monocytes	3.0	per cent
Platelets	140,000.0	
Mean cell diameter	7.4	
Plasma potassium	20.6	mg. per cent

The results are graphically represented in Charts 1 to 4.

\*Corrected for 0.5 c.c. plasma removed.

### DISCUSSION

*Red Blood Cells.*—The maintenance of the red blood cell counts at approximately a constant level over a period of a month suggests that, in the steady increase of the potassium content of the plasma, cell destruction plays at most only a small part.

Durán Jordá and his co-workers<sup>3</sup> reported there was a loss of 1,500,000 erythrocytes over a period of sixteen to twenty days in blood conserved in citrate. They suggested the stroma of these destroyed cells might play a part in some of the slight posttransfusion reactions. We have not found changes as

## RATE OF POTASSIUM DIFFUSION IN HEPARINIZED BLOOD

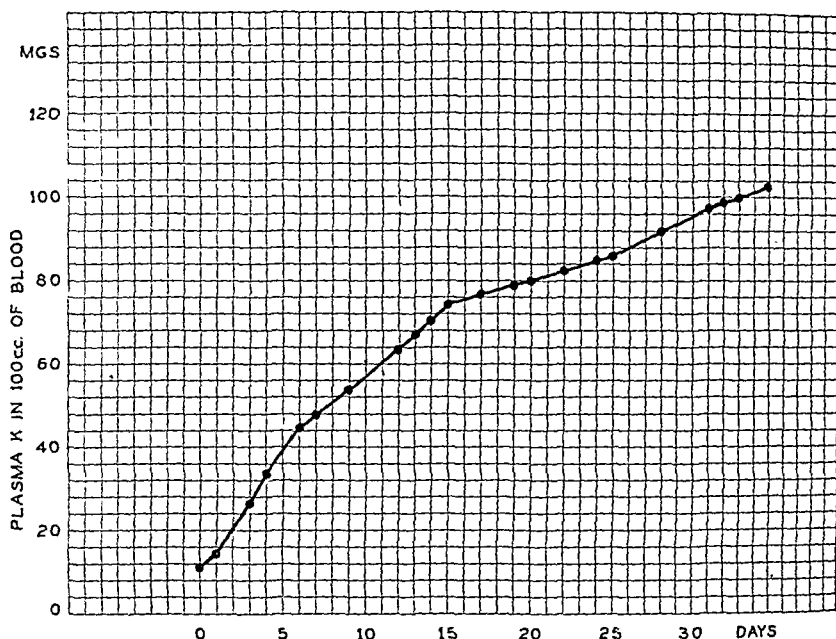


Chart 1.—Potassium diffusion in heparinized blood. Each point on the chart represents a determination done on different test tube samples each day and is calculated to represent the actual amount of potassium in milligrams in the plasma of 100 c.c. of blood. The values are approximately the same as in previous experiments where daily determinations were done on the same blood with the final curve representing a summation of the increments.

## CELLULAR CHANGES IN HEPARINIZED BLOOD

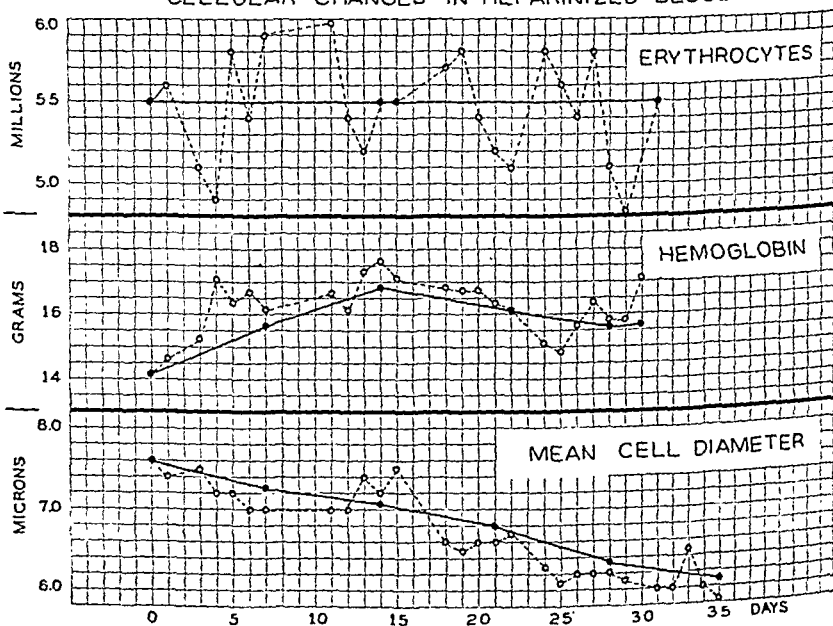


Chart 2.—Upper third. Red cell counts varied between 6,200,000 and 4,800,000, the mean being 5,500,000. No actual loss in number of red blood cells.

Middle third. Hemoglobin values varied between 14.2 and 17.6 gm. per cent. The curve, allowing for slight concentration due to evaporation, represents a constant hemoglobin content. The drop after the fifteenth day may be accounted for by the gradually increasing amounts of hemoglobin removed in the plasma taken for potassium determinations.

Lower third. There was a gradual decrease in the size of the red blood cells from an initial 7.6 microns to a final 5.8 microns as the cells lost both hemoglobin and potassium.

great as they report. The fact that all of our tubes were centrifuged for an hour before each count may play some part in this discrepancy.

Yudin,<sup>4</sup> from Russia, has reported that there is no loss in ability of cells so stored to carry oxygen, hence the chief biological function of the red blood cells seems unimpaired.

*Hemoglobin.*—The hemoglobin content remains constant. This confirms the findings of previous writers. The work of Amberson<sup>5</sup> has indicated that the part of the hemoglobin which escapes from the cells to give the usual picture of hemolysis is still capable of functioning normally.

#### CELLULAR CHANGES IN HEPARINIZED BLOOD

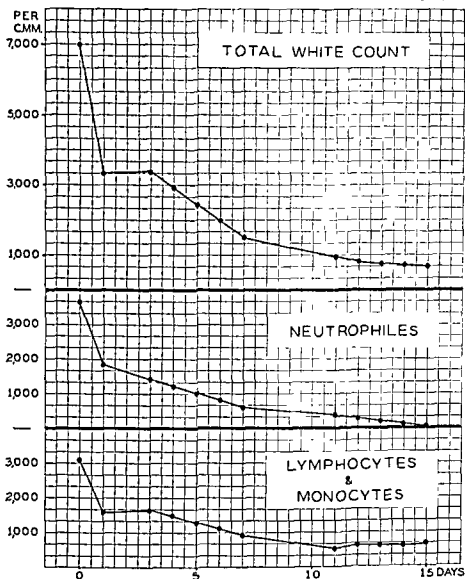


Chart 3.—*Upper third.* The total white blood cell count fell 50 per cent in twenty-four hours, the cells lost shape and gradually disappeared by the sixteenth day, those remaining appearing only as gelatinous-like clumps. Range 7,000 to 675 cells.

*Middle third.* The polymorphonuclear leucocytes showed earliest and most rapid changes; their nuclei soon losing shape and disintegrating. Range, 3,600 to 11 cells.

*Lower third.* Lymphocytes and monocytes disappeared at a slower rate and retained their shape, size, and staining better than the leucocytes, being quite distinct when found on the thirtieth day. Basophilic and eosinophilic cells, while too few to be charted, retained their shape, size, and staining qualities. The latter were least changed and very distinct on the thirtieth day. Range, 3,100 to 654 cells.

*Volume Index and Diameter of Red Blood Cells.*—The volume index of the cells decreases in successive specimens. There is a diminution in white blood cells, but the greater part of the decrease seems due to the diminution of the size of the red blood cells. At the end of thirty days these cells may lose as much as 25 per cent of their chief base, 20 per cent of their hemoglobin, and decrease 20 per cent in diameter. Ponder<sup>6</sup> has shown that red blood cells do not

always act as perfect osmometers, a fact which he attributes to the possible loss of their salt content.

*White Blood Cells.*—The total white blood cell count rapidly diminishes with the polymorphonuclear leucocytes disintegrating most rapidly. The reported loss of half of the complement of the blood by the sixth or seventh day, and complete destruction in fifteen to twenty days, may be associated with this loss of leucocytes.<sup>3</sup>

The lymphocytes are more resistant. At the end of thirty days they are easily recognizable when found, but there are so few that counts are uncertain. This holds true, likewise, for monocytes.

The eosinophiles appear most resistant. The nucleus breaks up late and the granules remain unusually well defined.

Durán Jordá<sup>3</sup> has reported similar findings.

#### THROMBOCYTES IN HEPARINIZED BLOOD

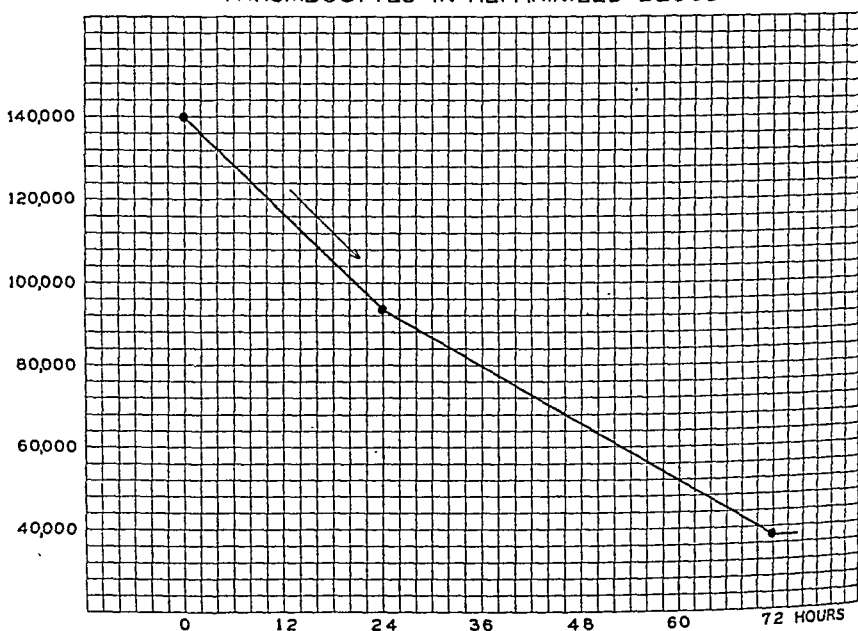


Chart 4.—Thrombocytes rapidly fell to about 40,000 and for thirty days remained approximately at this figure. Only the first three days are charted.

*Thrombocytes.*—The platelets in the initial venous blood sample numbered 140,000. These rapidly diminished in the first three days and remained at about 30,000 for fifteen days. Centrifugation may have some part in these counts. This suggests, however, that syndromes presenting thrombocytopenia would not be benefited by blood which had been stored too long.

*Potassium.*—The rate of potassium loss from the cells in the individual test tubes is of the same order as that found in the flasks from which daily samples were taken. The potassium content of white blood cells (those obtained from the buffy coat in case of lymphatic leucemia) is greater than the red blood cells. In normal blood their relative number is insignificant. However, their early rapid destruction may account in part for the steep slope of the curve during the first few days.

## CONCLUSIONS

1. In heparinized preserved blood there is little or no actual loss in the number of red blood cells over a period of thirty days.
2. The hemoglobin content remains constant in the total sample, though 15 to 25 per cent may be found in the plasma.
3. The mean cell diameter of the red blood cells is reduced approximately 20 per cent in thirty days.
4. The polymorphonuclear leucocytes are diminished by 50 per cent in forty-eight hours and are amorphous masses in fifteen days.
5. The lymphocytes, monocytes, and eosinophiles do not disintegrate so rapidly, the latter are particularly well preserved.
6. The thrombocytes rapidly fall to a low level, then remain constant at about 30,000 for about fifteen days.
7. Potassium in the plasma of heparinized blood reaches a level ten times normal in thirty days.

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## GLOMUS TUMOR OF ARM\*

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TO DATE 106 cases of glomus tumor have been reported in the literature. It is our purpose to add a case in which the tumor occurred at the site of a pre-existent "blue birthmark" and whose onset showed a suggestive relationship to trauma.

The present concept of the glomus is based upon the work of Suequet, Hoyer, Grosser, Schumacher, Masson, Clara, Popoff, and others. A glomus unit includes the following components: (a) an afferent artery, (b) Suequet-Hoyer canal, (c) neuroreticular and vascular structure around the Suequet-Hoyer canal, (d) an outer lamellated collagenous tissue, and (e) a primary collecting vein. This unit comprises the arteriovenous anastomosis.

The most constant sites of the normal glomus are in the hands and feet. Here they may be found in the ventral surfaces of the hands and feet, in the

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region of the nail beds, in the tips of the digits, in the palmar surfaces of the first, second, and third phalanges, and in the thenar and hypothenar eminences of the hand, and in the sole near the heel. Investigation has also disclosed them to be present within the bones of the terminal phalanges, the skin, lips, nose, eyelids, tip of tongue, tendons, gums, pia mater, dura, iris, ciliary bodies and endocardium. These glomic units are found in a definite zone of the corium, just below the web of subpapillary arteries and veins.

Popoff was unable to find them in fetuses aged 4½ months to term. Their development occurred during the first two months of postnatal life. He also demonstrated that over the age of 60 there is a diminution in the number of Suequet-Hoyer canals per square centimeter. This decrease is progressive with increasing age. They are absent in supernumerary digits. Grosser was unable to find them in reptiles, but he did find them in many mammals.

The ascribed function of the glomus body is to contribute to the control of the temperature-regulating mechanism of the body and to coordinate with the mechanism of peripheral vascular pressure control. When there is exposure to cold, the glomic system maintains and even raises local temperature. This is accomplished by diverting blood from the capillaries into collecting veins which have a highly developed surface area. When the glomic system is fully opened, it aids the dispersal of heat by allowing an enormous flow of blood to pass through the digits. When closed, it reduces the dispersal of heat. With an elevation of peripheral pressure it is capable of diverting arterial blood and thereby relieving the arterial system of a part of its burden. The absence of the glomic system during fetal life and its somewhat tardy development post-natally may be contributory to poor control of body temperature in premature infants and some newborns.

The most common aberration reported clinically is tumor formation. As such it occurs in sites where glomic bodies are normally found, the extremities being the most common sites. Although it is usually a single tumor, two cases of multiple tumors have been described by Bergstrand. Radach found four other reports of multiple tumors. The age occurrence is between 20 to 80. One case has been reported in a child of 6. The shortest duration of all the tumors was six weeks, the longest was 37 years. Subungual glomal tumors usually were noted at an average age of 25. Tumors at other sites were noted at an average age of 41. The outstanding clinical symptom was pain, intermittent or constant. Weidman and Wise have, however, reported a case in which they found multiple "glomic tumors" which were painless. Histologically their case showed chiefly cavernous dilatation and no nerve involvement. They considered their tumors to be a microscopic varicosity of a glomus rather than a true tumor.

Most of the tumors revealed no clinical evidence of any etiologic factor. Several observers were able to elicit a suggestive relationship to trauma. Slepian reported finding two such cases. Jirka and Senderi elicited a history of trauma. Our case gives a history of trauma.

The glomal tumor has been accredited benign, and to date no glomal tumor occurring at one of the usual sites has been reported otherwise. Stout, report-

ing a series of 11 cases, noted a recurrence at the original site eight years after removal. Recently, Kirshman and Teitelman, reporting a fatal metastasizing tumor of the omentum, described a histopathologic picture simulating a glomangioma. Since no normal glomic bodies have ever been described in the omentum, they raise the question of origin.

All of the elements of the Sucquet-Hoyer canal and its surrounding components have been designated as being the origin of the tumors cells.

Jirka and Seuderi implied an endothelial origin to the tumor cells. Krompecher considers them as angioblasts. He was unable to demonstrate myofibrils. Geschickter, because of the method of termination of the neurofibrils, believed the glomal cells to be modified nerve cells. Popoff, in his study of normal glomus, found amid the ordinary muscle cells, large epithelioid cells with clear cytoplasm and round or globular nuclei. Followed in serial section, these epithelioid-like cells appeared to be transversely cut muscle cells with voluminous cytoplasm and slightly elongated polyhedral nuclei.

Popoff, further in his study of the digital vascular system, had noted primary pathologic changes of the glomic unit in nonspecific cellulitis, senile arteriosclerotic gangrene of the foot, and diabetic gangrene. In thromboangiitis obliterans the pathologic changes were secondary.

The patient, a white male, aged 42 years, complained of a painful nodule on the left arm. He stated that a small nodule had been present above the elbow for ten years as a "blue birthmark." It had never been painful. Eight months prior to admission, while putting his arm out of an open automobile window, he struck the region of the nodule against the frame. At that time he states that it felt like a nail had been stuck into his arm. From then on he had a constant dull ache at the site of the injury. About two months after the injury he began to experience episodes of intermittent pain at the site of the nodule. These pains he described as rapidly succeeding paroxysms as though a needle was stuck into his arm." Each episode was associated with a similar pain, located at the level of the left sixth rib at the anterior axillary line. The nodule was painful when pressed. He noted no increase in size. Local examination revealed a small blue area,  $\frac{1}{4}$  inch in diameter, on the posterior aspect of the left upper arm, 8.5 cm. above the olecranon. The nodule, although sensitive to pressure, revealed no "trigger tenderness." The remainder of the physical examination, except for several small scattered hemangiomas on the anterior chest wall, was of no significance. The blood Wassermann and Kahn tests were negative.

*Gross:* The specimen consisted of an oval piece of skin, 1.5 cm. in long diameter, covering a small amount of subcutaneous tissue. In this tissue was found a small, lentil-shaped, dark brown, sharply circumscribed nodule.

Low-power examination revealed a nodule bordered by a well-defined fibroelastic capsule. The nodule was formed by intercommunicating vessels. Each vessel was surrounded by a mantle of spheroidal cells. These units were separated by small areas of collagenous tissue. Under higher magnification the endothelium of the vessels consisted of elongated cells resting on a collagenous membrane which varied in thickness. With the van Gieson's stain the collagenous fibrils were noted to radiate from the vessels and showed a dendritic proliferation between the tumor cells. Stained by the Bielschowsky-Maresch method, the connective tissue fibrils were deep black on a clear background. Thick collagen bundles were reddish brown.

The cells were in single and in group arrangement and appeared related to the collagenous fibrils as the fruits of a laden-down tree to its branches. The tumor cells about the vessels were distributed in fantastic waves in a more or less pyriform arrangement. The cytoplasm was ill defined and faintly staining. From it projected slightly elongated processes. The nuclei were stained deeply and contained granular chromatin material. No mitotic figures





Fig. 1.—Low power.



Fig. 2.—Medium power.

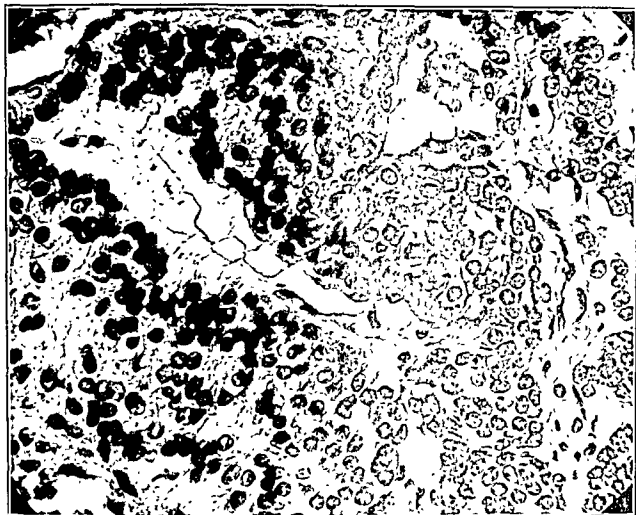


Fig. 3.—High power.

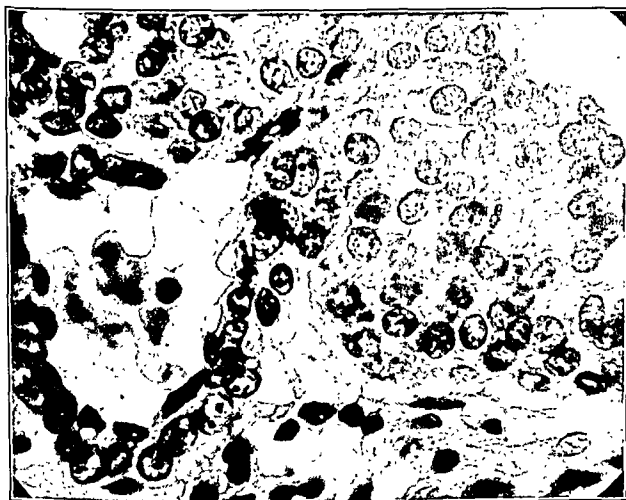


Fig. 4.—Oil immersion.

were noted. Sections stained by Bodian's method disclosed the presence of nonmyelinated nerve fibers which appeared to be in continuation with some of the foregoing cellular processes. Between the tumor cells in the collagenous tissue occasional fibroblasts and fibrocytes could be seen.

In accordance with other observers and with the classical work of Masson, we believe that the tumor cells originate from the muscular elements of the Suequet-Hoyer canal and that they are classified as myoblasts. We further believe that these cells are connected to the periglomal nerve elements by nonmyelinated nerve fibers.

#### SUMMARY

A case of glomus tumor occurring in a 42-year-old white male is reported. The tumor occurred at the site of a previous "blue birthmark," following injury to that site. The "birthmark" was of ten years' duration, and the glomus tumor was of eight months' duration. The tumor cells appear to be of myoblastic origin. We suggest that there exists a connection of these cells to the periglomal nerves by means of nonmyelinated nerve fibers. In view of the fact that the exact nature of the previous "blue birthmark" is unknown, it is difficult to postulate a relationship of the glomus tumor to it, although some relationship is extremely suggestive. We believe that the glomus tumor is a type of angioma, a complex type, in which the neuromyoblastic elements play an important role for clinical manifestations and for histologic morphology.

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# EFFECTS OF INTRAPERITONEAL INJECTIONS OF STAPHYLOCOCCUS ANTITOXIN ON SUBCUTANEOUS STAPHYLOCOCCIC INFECTION IN MICE\*

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THE mechanism of staphylococcus immunity, both local and general, is not clearly understood. Much emphasis, however, has been placed upon antibacterial immunity. Wright and Douglas<sup>1</sup> thought that opsonins played the important role in local immunity. It is now well known that staphylococcus vaccines produce an increase in the opsonic index, and the animals show a greater resistance to staphylococcus infections. Cannon and Pacheco,<sup>2</sup> in their study on immunity, immunized guinea pigs with a staphylococcus vaccine and then injected a virulent culture of the same bacterium subcutaneously. Histologic sections of the skin of these animals showed that the organisms formed clumps and clusters. A greater number of leucocytes and more phagocytosis were present in the sections from the immune pigs than in the normal controls.

Recently attention has been directed toward antitoxic immunity in staphylococcus infections.<sup>3-5</sup> The specific antitoxin does protect rabbits against a lethal dose of staphylococci. This protection is thought to be the result of a neutralization in vivo of the toxin by the antitoxin.<sup>6</sup> Investigators, in studying this immunity, have been concerned primarily in obtaining complete protection against the lethal action of cultures of staphylococci following an intravenous injection. Little attention has been given to the effects of staphylococcus antitoxin on local staphylococcus infections.

The present study was made to determine the type of reaction that would occur in the subcutaneous tissues when toxin- and nontoxin-producing strains of staphylococci were injected locally in normal mice and mice passively immunized with staphylococcus antitoxin. Staphylococcus toxin was also injected into a group of the normal mice to compare the reaction produced by the toxin with that produced by the bacterial cells.

## MATERIALS AND METHODS

The staphylococcus toxin† used in this study contained at the time of its preparation 8,000 dermonecrotic units per cubic centimeter. It was diluted 1:100 with physiologic saline immediately preceding the inoculation. Injections of 0.05 c.c. of this diluted toxin were made subcutaneously into each side of the abdomen of 20 white mice.

The toxin-producing strain was an *aureus* variant from a culture obtained in May, 1933, from the pharynx of a patient with agranulocytic angina. The

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†The toxin was supplied through the courtesy of Lederle Laboratories.

lethal effect of this variant on rabbits was recently reported.<sup>6</sup> The ability to produce toxin was determined by the method of Biggers.<sup>8</sup> The supernatant fluid obtained from washing a forty-eight-hour growth from an agar slant (15 by 2.5 cm.) contained enough hemotoxin in 0.05 c.c. to lyse completely 2 c.c. of a 1 per cent suspension of rabbit red blood cells. The nontoxin-producing strain of *Staphylococcus albus* was isolated in May, 1937, from a furuncle in the nose. No lysis of the red blood cell suspension occurred when 0.8 c.c. of the supernatant fluid from this culture was added.

The staphylococci were grown on agar slants for eighteen to twenty-four hours immediately preceding the animal inoculation. The washed bacteria were suspended in a physiologic salt solution. The suspensions of the organisms were brought to approximately the same concentration. Injections of 0.05 c.c. of these suspensions were made subcutaneously on each side of the abdomen of white mice. Twenty were given the suspension of the nontoxin-producing strain, and 26 the suspension of the toxin-producing strain. Two mice from each of three groups, namely, those receiving the toxin, the toxigenic and nontoxigenic strains of staphylococci were killed after thirty minutes, one, one and one-half, two, three, four, five, six, eight, and ten hours. Two mice inoculated with the toxin-producing strain were also killed after eleven and twelve hours.

Mice were passively immunized by two injections of 0.5 c.c. of staphylococcus antitoxin\* given intraperitoneally at eighteen hours and at two hours prior to the subcutaneous inoculation of the washed bacteria. Groups of four of these immune mice were killed after intervals of three, six, and ten hours.

A 27 gauge needle was used for inoculating the bacteria. The abdominal wall in the area of the lesion was removed and placed in a 5.0 per cent solution of Zenker acetic acid immediately after the animals were sacrificed. The sections were prepared and stained with hematoxin and eosin. Select sections were stained according to Giemsa's method.

#### EXPERIMENTAL

*Lesion Produced by Staphylococcus Toxin in Normal Mice.*—The mice inoculated with the staphylococcus toxin appeared slightly less active than the controls. All of them survived the effect of the inoculum and were killed. The abdominal wall in the area of the inoculation was macroscopically edematous and hyperemic after three to four hours. The blood vessels were dilated on the peritoneal surface. Microscopically the vessels were dilated after thirty minutes. At this time the subcutaneous tissues were edematous and remained swollen during the remainder of the experiment. The muscle in the abdominal wall showed focal hyaline and nonstriated areas, and necrosis after ninety minutes. The injury to the tissue apparently did not progress after the first two hours. Polymorphonuclear leucocytes were the characteristic cells present. After thirty minutes these cells lined the lumina of the dilated vessels, and were present in the adjacent tissue. Only a few leuco-

\*Supplied through the courtesy of Lederle Laboratories.

cytes were found in the edematous and necrotic tissue during the first three hours after inoculation. Many of these cells were pyknotic and lyzed. After six hours the leucocytes appeared more numerous and better preserved. At this time they seemed to be in greater number about the necrotic muscle tissue.

*Lesions Produced by a Nontoxigenic Strain of Staphylococci in Normal Mice.*—The mice appeared normal following the inoculation. The early gross lesions were similar to those which occurred with the toxin. The bacteria were more or less localized in the subcutaneous tissue. The muscle never showed any evidence of necrosis, and the subcutaneous tissue showed only a small amount of necrosis at the end of the experiment. Polymorphonuclear leucocytes were the characteristic cells in the inflammatory process. After thirty minutes these cells lined the lumina of the dilated vessels and infiltrated the adjacent tissue. As time progressed the leucocytes appeared to migrate toward the bacteria. After three hours the leucocytes had collected about the organisms and infiltrated the adjacent tissue. After eight hours the leucocytes had invaded the entire area previously occupied by the bacteria, and practically all the latter were phagocytosed. Little change occurred in the reaction during the last two hours of this experiment.

*Lesions Produced by a Toxigenic Strain of Staphylococci in Normal Mice.*—A majority of the 26 mice that received these organisms were weak and would not eat after three hours. Four of the group died five to ten hours after the injection. Pathologic studies were made only on the mice that were sacrificed.

Macroscopically the abdominal wall in the inoculated areas was edematous and hyperemic after three hours. As the experiment continued, the edema progressively spread to involve the greater portion of the wall. After six to eight hours the skin was pale yellow in color. Microscopically the changes after thirty minutes were similar in only a few respects to those in which the nontoxin strain of staphylococci was injected. Polymorphonuclear leucocytes were present in the tissues about the blood vessels in the areas where there were no bacteria. Only a few leucocytes apparently ever infiltrated the zone occupied by the organisms, and these were destroyed very rapidly. Phagocytosis was not a conspicuous process at any time during this experiment (Fig. 1C).

Although it was impossible to completely control the site of inoculation, there seems to be no doubt that the bacteria invaded very quickly the surrounding tissue. The leucocytes failed to localize the organisms after eight hours as they did the nontoxin-producing strain of staphylococci. Extensive necrosis occurred after five hours (Fig. 1A). Bacteria invaded the walls of some of the smaller blood vessels. Injured and degenerated leucocytes infiltrated the tissue in the abdominal wall. Staphylococci and leucocytes were present along the peritoneal surface.

*Lesions Produced by a Nontoxigenic Strain of Staphylococci in the Immune Mice.*—The mice were weak and had severe rigors within five minutes after receiving the staphylococcus antitoxin. After two hours they appeared nor-

mal. Three hours after the inoculation of the bacteria the abdominal wall was edematous and hyperemic. This reaction slowly progressed during the remainder of the experiment.

The histologic changes in these mice were very similar to those in the normal. Some of the bacteria appeared to form small clusters. It is difficult to determine whether or not there was greater agglutination of the bacteria

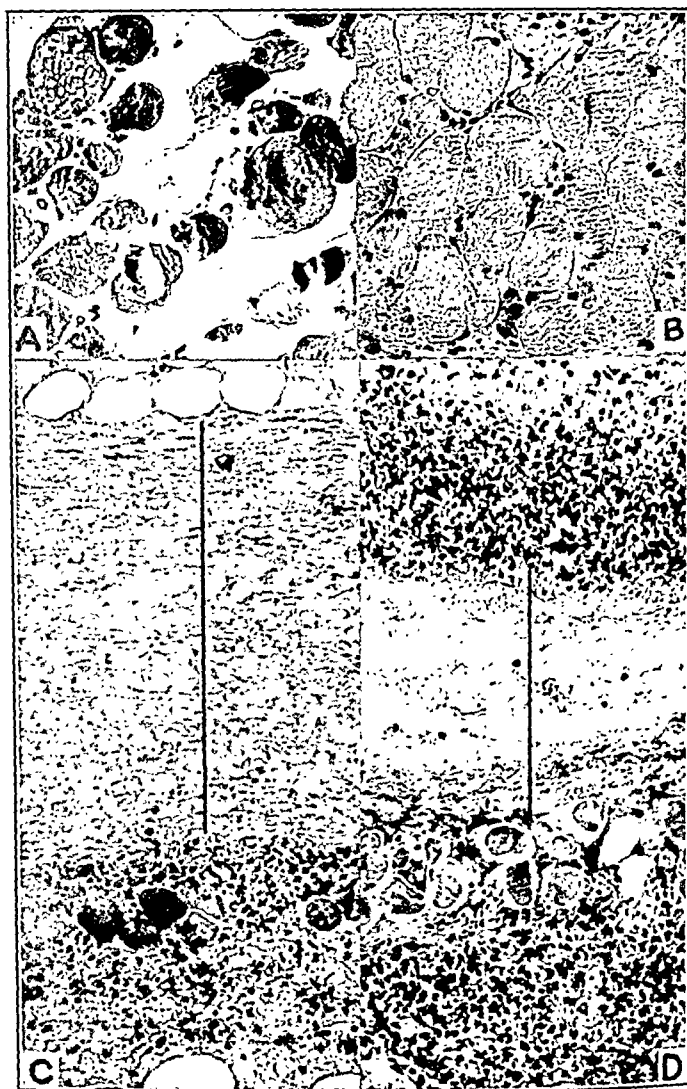


Fig. 1.—A, The abdominal muscle is severely injured by the toxin liberated by the staphylococcus in vivo. This normal mouse was killed five hours after injecting subcutaneously 0.05 c.c. of a toxigenic strain of staphylococci.

B, The abdominal muscle is normal in the passively immunized mouse six hours after receiving the same suspension of bacteria as shown in A.

C, Only a few polymorphonuclear leucocytes are present after five hours about the toxin-producing staphylococci in the subcutaneous tissue of this normal mouse. The bacteria are present in the greatest number in the subcutaneous tissue in the zone indicated by the line.

D, The bacteria are more localized and there is a marked increase of leucocytes in the subcutaneous tissues of this passively immune mouse when compared with the animal shown in C. A majority of the staphylococci are present in the subcutaneous tissue in the zone indicated by the line. Section removed after six hours. (Hematoxylin and eosin.  $\times 275$ .)

in the immune than in the normal mice. There were many more polymorphonuclear leucocytes, and there was greater phagocytosis in the immune animals than in the normal mice.

*Lesions Produced by a Toxigenic Strain of Staphylococci in Immune Mice.*

—The mice in this group looked very similar to those receiving the nontoxin-producing strain. Macroscopically the lesions in the two groups of immune animals were very much alike. Microscopically the toxin-producing strain of staphylococci never invaded the adjacent tissues as extensively as they did in the normal. Three hours after injecting the bacteria there were many polymorphonuclear leucocytes present. Apparently there was also less destruction of cocci and greater phagocytosis of the bacteria by the leucocytes in the immune animals than in the normal (Fig. 1D). These toxin-producing organisms remained localized in the immune mice the same as the nontoxin-producing strain. At no time was there extensive edema and necrosis like that which occurred in the normal mice (Fig. 1B). The only necrosis observed in the muscle was found in a few areas where there was an extraordinary large number of staphylococci.

#### DISCUSSION

The absence of any polymorphonuclear leucocytes in the tissues of normal mice for approximately three hours after the injection of staphylococcus toxin, and the presence of local necrosis during this time may indicate either that the leucocytes are not present in the tissue or that they are destroyed by the toxin. During the first three hours following the subcutaneous inoculation of toxin, apparently all of it either unites with the tissue or is removed by the blood or lymph. Leucocytes apparently are not destroyed by the toxin after this time as they enter the areas to phagocytose the cellular debris resulting from the action of the toxin on the tissue.

The toxigenic strain of staphylococcus produces lesions in the normal mice similar to those produced by staphylococcus toxin alone. In view of this it appears very likely that the staphylococcus liberates a substance in vivo which injures the tissues. The effects of this substance are not as evident when animals are passively immunized with staphylococcus antitoxin.

In the absence of staphylococcus antitoxin the toxin-producing strain invades the necrotic tissue. The toxin liberated in vivo produces necrosis of the tissues and destroys the leucocytes. In passively immune animals the bacteria apparently remain localized, and they are more quickly phagocytosed. The toxin liberated is evidently immediately neutralized by the antitoxin.

The reaction of the tissues to a nontoxin-producing strain of staphylococci is very similar in both the normal and immune mouse. There may be, however, more leucocytes and greater phagocytosis in the latter. It is difficult to determine from histologic studies whether or not there is an increase in the number of leucocytes since there is some variation in normal mice. There are many observations, however, which show that there is greater phagocytosis of bacteria in animals immunized with vaccines than there is in the normal.<sup>9, 10</sup>



The observations made in this study on the local tissue reactions with toxin and nontoxin-producing strains of staphylococci are similar to those of Tsuda using virulent and avirulent streptococci and pneumococci.<sup>10</sup>

There are a larger number of leucocytes and a greater phagocytosis in the immune than in the normal mice. Apparently the antitoxin neutralizes the toxin and thus prevents the destruction of the leucocytes. The increased phagocytability may be the result of a longer survival of the leucocytes permitting them to continue to phagocytose the bacteria.

Staphylococci are present in clusters in the mice given staphylococcus antitoxin. Similar groups of bacteria are also observed in some of the normal animals. This clumping of the bacteria may have resulted from inoculating groups of organisms directly into the tissue. Staphylococci, as is well known, have a tendency to grow in groups in vitro.

Ramon,<sup>3</sup> in discussing the effect of staphylococcus antitoxin, expressed the opinion that it protects the cells and tissues against the necrotizing action of the staphylococcus poison and thus makes a less favorable medium for the growth and multiplication of the organisms. The leucocytes, in the presence of antitoxin, are able to phagocytose the bacteria and debris. Staphylococcus antitoxin is, therefore, indirectly bactericidal. The results of this experiment confirm Ramon's opinion and indicate that greater phagocytosis of a toxigenic strain of staphylococci occurs in mice passively immunized with staphylococcus antitoxin than occurs in normal mice.

The mechanism of the immunity produced by staphylococcus antitoxin in local staphylococcus infections appears to be primarily the result of the neutralization of the toxin. The leucocytes apparently survive for a longer period in the immune animal and continue to phagocytose the bacteria. Staphylococci may also be more easily phagocytosed in the presence of immune sera. The importance of leucocytes in staphylococcus immunity has been shown by Cannon and Pacheco<sup>2</sup> in animals immunized with staphylococcus vaccines. The present study also shows the importance of leucocytes in animals passively immunized with staphylococcus antitoxin.

#### SUMMARY

Mice passively immunized with staphylococcus antitoxin and given staphylococci subcutaneously show more polymorphonuclear leucocytes and greater phagocytosis than the controls without antitoxin. If the staphylococcus produces a toxin in vivo it is neutralized by the antitoxin. The toxin-producing strains of staphylococci remain localized in the immunized mice similar to a nontoxin-producing strain in normal mice.

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## GERMICIDAL PROPERTIES OF THE OXIDES OF NITROGEN\*

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SO FAR as we have been able to determine, there has been no work published in literature that has detailed the systematic study of the germicidal action of the oxides of nitrogen.

Review of the literature does reveal that the oxides of nitrogen have been used with excellent results in the treatment of infected wounds, osteomyelitis, chronic empyema, and tuberculous sinuses. Much of this work has been done and published by Sweek,<sup>1</sup> of Phoenix, Ariz. He and others have been fully aware of the remarkable bactericidal properties of the oxides of nitrogen but have not taken up the work involving a purely bacteriologic study.

In 1903, J. N. Alsop, of Owensboro, Ky., experimented with a machine for aging flour. This machine consisted mainly of a high tension electric arc, setting up vibrations in the air. During his experiments he occasionally noticed dead flies around the machine. Brockett, of Princeton, discovered that the gas from a similar machine possessed definite bactericidal powers. Alsop cured dogs of mange by placing them in a chamber containing the electrified air.<sup>2</sup>

Dr. Sternon, of Owensboro, Ky., devised and used a machine in which a pine block was placed in the ionizing chamber and inserted into the electric arc at intervals. This type of machine was used also by the late J. B. Murphy and his associates at the Murphy Clinic. It was thought that the pine block would add certain organic matter to the gas which would enhance its bactericidal power.

Dr. Lowell, at the Mercy Hospital in Cincinnati, now uses a machine in which the gas comes in contact with a pine stick. He believes that the organic matter derived from the pine is an essential factor in preparation of the germicidal gas.

\*From the Lois Grunow Memorial Clinic, Phoenix.  
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Dr. W. O. Sweek substituted pine oil for the pine block. Air was passed over the pine oil before entering the ionizing chamber. He, however, discarded the idea of the organic matter several years ago, as he has seen no difference in the bactericidal power nor any difference in the rapidity with which infected wounds heal. He now uses a machine in making the gas which does not bring organic matter in contact with the arc.

In our experiments we have used a duplicate of Dr. Sweek's machine, a description of which is included in this report.

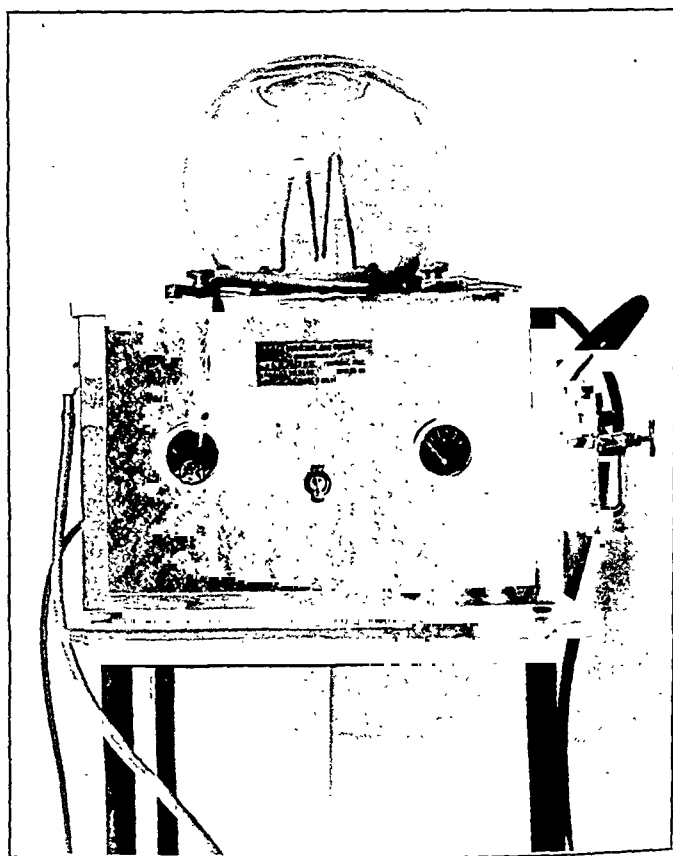


Fig. 1.—The gas machine used in the experiments. It will be noted from its description that it is very compact and easy to transport. While the machine is in operation, it is more expensive to operate than an ordinary electric iron.

In taking up the study of any antiseptic or germicide for general use, one must consider several factors; namely:

1. The availability and economical status of the antiseptic.
2. What harm can be done to a patient or tissues with which it comes in contact during its administration?
3. Does it possess definite antiseptic and bactericidal qualities?

In answer to the first factor we can safely say that it is easily and economically produced. The second factor has been discussed in previous publications.<sup>1</sup> However, we may state here that this gaseous antiseptic correctly

used within its limitations is harmless. The discussion and study of the third factor will be the purpose of this paper.

For a more thorough understanding of the antiseptic under consideration, we give a brief description of the preparation of the gaseous antiseptic used in these experiments.

When compressed air is passed through an electric arc of sufficient amperage and voltage, the oxygen and nitrogen of the air undergo a certain chemical combination. The exact sequence and the end products of these changes are not definitely known. However, we do know that the oxides of nitrogen are produced and that the resultant gas, in the presence of moisture, forms nitric acid. We know also that when an equilibrium is reached, the percentages of the different oxides of nitrogen vary with several conditions; namely: (1) the amperage and voltage used; (2) the width of the spark; (3) the pressure of the compressed air as it enters the ionizing chamber; (4) the distance from the arc at which the gas is taken; and (5) the temperature of the gas. Variations in these factors have a very definite influence on the germicidal qualities of the gas as well as its effect on the tissue cell.

We believe that the machine we have used in our experiments has the above factors so adjusted as to have the maximum power as a germicide with a minimum effect on the tissue cell.

Fig. 1 shows the machine used in evolving the germicidal gas used in our experiments. The dimensions of the cabinet are  $16\frac{1}{2}$  by  $10\frac{1}{2}$  by 10 inches. Air enters through the right end panel. The gas outlet is through the left end panel and the electrical attachment cord. The top panel carries a 10 liter ionizing chamber, air nozzle, and electrodes. Air at 2 to 3 pounds pressure enters through the right end of cabinet and proceeds on into the ionizing chamber through  $\frac{1}{8}$  inch air nozzle directly below electrodes. The amperage is 60 Ma. at 12,000 volts. As the air passes through the arc, the oxygen and nitrogen undergo certain chemical combinations. It makes its escape from the ionizing chamber through an outlet at the left end of the cabinet. At the end of a  $\frac{5}{16}$  inch 10 ft. rubber hose, a glass nozzle or catheter is attached for treatment purposes.

The clinical germicidal and fungicidal effect of the gas having been demonstrated in many cases, it was, of course, obvious to us that these qualities could be demonstrated in the laboratory by bacteriologic experimental methods. We have repeatedly demonstrated this to be true during the past several years by numerous and varied cultural experiments with many different kinds of bacteria and fungi. In every laboratory experiment the high germicidal and fungicidal effect of the gas was demonstrated even more convincingly than in the clinical cases treated.

Tables I to V show a series of carefully controlled bacteriologic experiments. These experiments were carried out with pure cultures of various bacteria obtained from Parke, Davis and Co. It was thought best to employ strains of bacteria obtained from outside sources in order to avoid the possibility of encountering weakened or attenuated strains accidentally isolated by us or resulting from numerous necessary subcultures while isolating pure strains in our own laboratory.

TABLE I

Inoculations were made from saline emulsion and forty-eight hours' agar growths. They were gassed and allowed to incubate twenty-four and forty-eight hours before the readings were taken. It will be noted that the *Streptococcus pyogenes*, which is ordinarily killed quickly, proved to be a good grower, as recommended by the pharmaceutical house.

BACTERIA BRAIN VEAL AGAR		15 SEC.	30 SEC.	45 SEC.	60 SEC.	75 SEC.	90 SEC.	105 SEC.	120 SEC.	135 SEC.	150 SEC.	CONTROL
<i>Staphylococcus aureus</i>		+	+	+	No growth	--	--	--	--	--	--	++++*
<i>Bacillus coli</i>		7 colonies	5 colonies	2 colonies	--	--	--	--	--	--	--	++++
<i>Bacillus pyocyaneus</i>		3 colonies	2 colonies	2 colonies	--	--	--	--	--	--	--	++++
<i>Streptococcus pyogenes</i>		++++	+++	+++	++	++	+	+	--	--	--	++++

\*Solid growth.

TABLE II

The strain of *Streptococcus pyogenes* is very resistant and blood agar cultures are more resistant to the gas on account of the lack of moisture. It will be noted also that the same strain was completely killed in one and one-half minutes in a saline suspension, as shown in Table III.

	5 MIN.	10 MIN.	15 MIN.	20 MIN.	25 MIN.	CONTROL
<i>Streptococcus pyogenes</i> 48-hour culture brain veal agar	+++	+++	++	--	--	++++

TABLE III

The bacteria are killed somewhat more quickly in the presence of more moisture.

	30 SEC.	1 MIN.	1½ MIN.	2 MIN.	3 MIN.	4 MIN.	5 MIN.	CON- TROL
<i>Streptococcus pyogenes</i> suspension in normal saline	++	+	2 colonies	--	--	--	--	++++

TABLE IV

Petri dishes were gassed and left open for twelve hours exposed to the air, then recapped, and incubated for twenty-four to forty-eight hours. It will be noted that the gas caused some change in the media that prevented growth of mold and bacteria. The Petri dishes were gassed five, ten, fifteen, twenty, and twenty-five minutes, respectively, before exposure to the air.

	5 MIN.	10 MIN.	15 MIN.	20 MIN.	25 MIN.	CONTROL
Petri dishes nutrient agar	--	--	--	--	--	++++

TABLE V

About five minutes of gassing of the Petri dish is necessary before the surface of the media becomes bactericidal.

	1 MIN.	2 MIN.	3 MIN.	4 MIN.	5 MIN.	CONTROL
Petri dishes nutrient agar	3 mold and bacteria	7 colonies bacteria	5 mold	4 mold	2 mold	Colonies of mold and bacteria

The *Streptococcus pyogenes* strain was obtained after the first strain furnished by them had proved to be lacking in growing qualities on subculture. The one used in this experiment was recommended by them as being a good "grower." We found that their prediction was accurate, and it will be noted that this culture was more resistant to the gas than any of the other strains of bacteria. It will also be noted, however, that all the bacteria were quickly killed by the gas. The media used in these experiments were the ordinary Difco products, the brain veal agar having a pH of 7.65 while the nutrient agar had a pH of 6.7+. It was interesting to note that even with prolonged treatment of the media by the gas the reaction of the media was not altered to the acid side sufficiently enough to be shown by any of the ordinary indicators. It is believed that this fact may somewhat explain the nonirritating quality of the gas when used over prolonged periods in the treatment of infected wounds and sinuses.

The Petri dishes used in this test were of standard size, and the agar slants were contained in test tubes  $\frac{5}{8}$  inch by 6 inches. The gas was applied to the surface of the slanted media through a rubber or glass tube with a tip small enough to reach entirely into the apex of the slant. If this was not done carefully, it was found that a pocket of air would be produced in this recess, preventing the gas from reaching the bacteria here. It was also found important to move the tip about slightly while applying the gas in order that no bacteria would be imprisoned underneath the tip, thus preventing access to them by the gas.

It has been suggested that the gas passing over the surface of the media might exert a drying effect, besides blowing away some of the bacteria. With this possibility in mind, a series of experiments were conducted. Air alone was passed over the surface of the media in the same manner and under the same conditions as obtained when the gas was used. There was a luxuriant growth on each tube, showing that the drying or blowing effect was not a factor in these experiments.

One is impressed throughout these experiments with the high bactericidal power of this gaseous antiseptic. There has been much speculation, and

many theories have been advanced as to what particular factor or group of factors is responsible for the germicidal power of the gas.

Dr. Lowell's theory is that the beneficial effects are due to electronic changes brought about in the tissues, resulting in a more normal state of electrical balance, thus enabling the tissues to fight infection more effectively. This explanation applies to wounds that are being treated with the gas and is not an explanation of its germicidal activity in bacterial cultures.

Dr. Sweek is of the opinion that its germicidal activity is due to the formation and deposition of nitric acid and other compounds as yet unanalyzed, the bacteria being destroyed by dissolution or injured to such an extent that death of the bacteria results.

It has been noted that the amount of nitric acid deposited on the media is very small as it does not change the pH enough to be shown by the ordinary indicators. We do know, however, that if this gas is passed through distilled water for one to two hours, the pH is changed considerably toward the acid side, as shown by titrable acidity. We also know that the gas is not effective in the absence of moisture, but is this not true of any gaseous, liquid, or semi-solid germicide? From our clinical experience we may add that after gassing an open wound for one hour there has not been enough nitric acid deposited to irritate or injure tissue cells. On the contrary, the wound heals rapidly and cultures from the wound are sterile after two or three such treatments. It has been stated by Dr. Leo Loeb, and corroborated by others, that small amounts of dilute acid enhance the action of the phagocyte and stimulate granulation tissue formation. Further, in the treatment of infected wounds with this gas, the small amount of acid formed helps to dissolve fibrin and other necrotic material that might retard wound healing.

It is possible that some nascent oxygen is formed in the gas which exerts its benefit as an oxidizing agent. We feel that small amounts of hyponitrous acid are formed which further enhance the oxidizing power of the gas.

#### CONCLUSIONS

1. Compressed air passed through an electric arc, as described in this report, produces a mixture of gases which is a very effective bacteriostatic and bactericidal agent.

2. The bacteriostatic and bactericidal action of this gas is effective in fifteen seconds' exposure.

3. This gas is also fungistatic and fungicidal.

4. The vegetative forms of bacteria and fungi are the least resistant to the gas, the spore forms being very resistant.

5. We believe that the bactericidal power of this gas is due to formation and deposition of minute amounts of nitric acid and other agents contained in the gas that as yet have not been analyzed.

6. The gas is easily and economically produced and should find its greatest field of usefulness in the treatment of infected wounds and chronic draining sinuses, particularly osteomyelitis and tuberculous sinuses.

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## ULTRAVIOLET IRRADIATION AND VITAMIN C METABOLISM\*

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WITH the advance of knowledge, interest in vitamin C has shifted from consideration of its importance as an antiscorbutic agent to a fuller appreciation of its function in the physiologic defense mechanism against various infections and intoxications. It is now common experience that most infectious diseases are accompanied by a loss of the vitamin from the body; moreover, it is often possible, experimentally and clinically, to enhance resistance or to hasten recovery by raising the vitamin levels through the administration of ascorbic acid.<sup>1</sup>

The significance of low-grade C deficiencies, or hypovitaminoses, in the epidemiology of infectious diseases is far from being understood; indeed, the very complexity of the problem presents great difficulties for systematic exploration. However, it remains an inviting task to attempt to correlate seasonal variations in susceptibility, as indicated by the periodic oscillations of mortality and morbidity, with cyclic changes of vitamin C metabolism. Unlike the mode of utilization of other vitamins that are stored in considerable reserves in the body, the vitamin C intake and outflow represents a delicately attuned system which undergoes continuous alteration and adjustment. The changes in this system may be measured directly by the degree of C saturation of blood and tissues, on the one hand, and by the rate of C elimination through peripheral secretions and excretions (milk, sweat, tears, saliva, and urine) on the other. That the maintenance of this balance is subject to seasonal influence is suggested by numerous observations which need hardly be related here in detail.<sup>2</sup> It is as yet uncertain to what extent these phenomena are brought about by exogenous or by endogenous factors, individually or collectively; but there are good reasons for assuming that, apart from differences in dietary intake, environmental conditions connected with the operation of climatic constants must play an important part.

Exposure of guinea pigs to high temperatures, for instance, is followed by a diminution of vitamin C in the liver, brain, and adrenals, and the ad-

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ministration of ascorbic acid is said to protect the animals against the lethal effect of heat damage.<sup>3</sup> Similar were the findings of Zook and Sharpless,<sup>4</sup> who not only observed a rapid depletion of C stores in animals with elevated temperatures but also showed that artificial fever reduces the urinary excretion of the vitamin in man. Exposure of guinea pigs to ultraviolet light caused either an increase or a decrease of the ascorbic acid content of the skin, depending upon whether irradiation was brief or prolonged (Castellini<sup>5</sup>); similarly, Wels's<sup>6</sup> experiments demonstrated an actual increase of the sulfhydryl groups in swine skin following ultraviolet raying. These results are in good agreement with the observation of Holtz and Woellpert<sup>7</sup> that the clinical symptoms of scurvy were milder in C deficient guinea pigs, which had received small doses of ultraviolet light, than in nonirradiated controls, even though both sets of animals presented the fully developed picture of scorbutic lesions. The effect of ultraviolet light on vitamin C metabolism in man has been investigated by several authors, but the results are not entirely in accord. While Schade<sup>8</sup> reported that vitamin C administration inhibited both the erythema and pigmentation resulting from ultraviolet irradiation, Urbach and Kral<sup>9</sup> obtained a definite light-protective effect only by combining vitamin C doses with local application of bergamot oil. Unpublished data by Dr. Irving S. Wright,<sup>10</sup> to which we may refer by permission of the author, indicate that the production of an extensive ultraviolet light erythema was followed by an abrupt retention of urinary vitamin C in one out of three subjects; this individual was red-haired, fair-skinned, and could tolerate much less ultraviolet light than the other two subjects. Drigalski,<sup>11</sup> on the other hand, reported no change in the rate of urinary excretion of vitamin C as the result of ultraviolet irradiation. Since ultraviolet light in excessive doses provokes a severe tissue burn, it is not surprising to note that both Stolfi<sup>12</sup> and Einhauser<sup>13</sup> observed a precipitous drop in the vitamin C levels of various organs—especially marked in the adrenal—in rabbits or in guinea pigs whose extremities had been scalded by immersion in hot water. Einhauser also described the lifesaving effect of cortin and vitamin C, either together or alone, when given to animals suffering from experimental burns. The above quotations suffice to delineate the scope of the work that has been done in this field and, at the same time, serve to emphasize the limitations of our present knowledge.

We have been particularly interested in the relationship that seems to exist between vitamin C metabolism and the effect of ultraviolet light in the production of disease, when irradiation transcends the threshold of physiologic stimulation. Clinical experience has amply demonstrated the potential danger of excessive doses of ultraviolet light in the production of critical changes in capillary fragility which may be instrumental in causing hemorrhage in vitamin C deficient patients suffering from pulmonary tuberculosis or gastric ulcers. But apart from such accidents, light damage may possibly play a more fundamental part in the pathogenesis of certain infectious diseases that reach an unexplained seasonal peak of epidemicity during

the summer months. Thus, the coincidence of sunburn following unrestrained exposure at beaches and swimming pools and the occurrence of cases of poliomyelitis are so frequent and almost pathognomonic that some epidemiologists have seized upon this sequence as indicating the water-borne nature of poliomyelitic infection; in doing so, the obvious fact was overlooked that the individuals bathed not only in the water but also in the sun. The suspected correlation becomes even more compelling when one contrasts the rarity of the disease in southern climes, where the even presence of radiation throughout the year provides for a protective tanning, with its massed incidence in northern latitudes in which the full force of summer sunlight must be borne by an unprepared skin. Conceivably, the apparent racial difference in natural resistance to poliomyelitis that separates the highly susceptible white races from the relatively less susceptible colored races (black and yellow), may in some measure be related to the abundance of pigment in the skin of the latter, acting as a protection against the hypothetical photodynamic injury.

#### EXPERIMENTAL

It was the object of this study to determine whether or not exposure of laboratory animals to ultraviolet irradiation was followed by a significant change in the vitamin C content of representative tissues. The animals employed in this work were albino guinea pigs, rabbits, and rhesus monkeys; the tissues examined were brain, liver, spleen, and adrenal gland. As the source of ultraviolet light we used a mercury vapor lamp, energized by high frequency currents (Lepel) and emitting a spectrum in which the longer ultraviolet waves, in the range from 2,800 to 3,100 angstrom units, predominate. The same lamp had been used in previous studies on the inactivation of poliomyelitis virus by ultraviolet light.<sup>14</sup> Animals, whose backs had been cleanly shaven, were placed under the lamp at a distance of about 30 cm. from the burner and exposed to the light for from thirty to sixty minutes. After intervals of one and eighteen hours, respectively, the animals were killed, and their organs examined for vitamin C content. For purpose of control, unrayed animals, which had previously been kept on the same dietary regime, were sacrificed simultaneously, and vitamin C titrations of their organs were carried out, side by side, with those of the organs of rayed animals. Preliminary diets were planned to cover the approximate range from inadequacy to adequacy. Duplicate animals were used in each test, pooling the organs of pairs of guinea pigs for vitamin C analysis. The amounts of reduced vitamin C in the tissues were determined by titration with indophenol (sodium 2, 6-dichloro-benzenoneindophenol, Eastman Kodak Co.). Because of its simplicity and accuracy we adopted the modified method of Bessey and King,<sup>15</sup> the experimental error of which is estimated between 5 and 10 per cent; technical details may be found in an earlier paper in which the same method was employed satisfactorily for the determination of vitamin C levels of monkey tissues.<sup>16</sup> The results of the various titrations are brought together in Table I which also gives additional data relating to the experimental setup.

TABLE I

## EFFECT OF ULTRAVIOLET IRRADIATION ON VITAMIN C LEVELS OF TISSUES

EXPERIMENT*	ANIMAL	DIET	DURATION OF IRRADIATION	INTERVAL AFTER RAYING	VITAMIN C CONTENT OF ORGANS (MILLIGRAM PER GRAM)			
					BRAIN	LIVER	SPLEEN	ADRENAL GLAND
1	Guinea pigs	Inadequate	Control	--	0.18	0.07	0.25	0.62
	Guinea pigs	Inadequate	Control	--	0.16	0.08	0.16	0.39
	Guinea pigs	Inadequate	Control	--	0.13	0.08	0.16	0.36
	Guinea pigs	Inadequate	Control	--	0.14	0.08	0.23	0.57
	Guinea pigs	Inadequate	30 min.	1 hour	0.16	0.09	0.22	0.92
	Guinea pigs	Inadequate	30 min.	18 hours	0.15	0.07	0.15	0.36
	Guinea pigs	Inadequate	60 min.	1 hour	0.13	0.09	0.21	0.46
	Guinea pigs	Inadequate	60 min.	18 hours	0.14	0.09	0.24	0.40
2	Guinea pigs	Adequate	Control	--	0.20	0.25	0.54	1.44
	Guinea pigs	Adequate	Control	--	0.17	0.19	0.34	1.02
	Guinea pigs	Adequate	Control	--	0.19	0.26	0.50	1.47
	Guinea pigs	Adequate	Control	--	0.17	0.16	0.41	0.96
	Guinea pigs	Adequate	30 min.	1 hour	0.20	0.25	0.50	1.35
	Guinea pigs	Adequate	30 min.	18 hours	0.16	0.13	0.32	1.09
	Guinea pigs	Adequate	60 min.	1 hour	0.19	0.28	0.53	1.42
	Guinea pigs	Adequate	60 min.	18 hours	0.17	0.16	0.37	0.98
3	Rabbits	Adequate	Control	--	0.19	0.12	0.52	1.76
	Rabbits	Adequate	Control	--	0.20	0.16	0.41	2.29
	Rabbits	Adequate	30 min.	1 hour	0.19	0.13	0.49	1.92
	Rabbits	Adequate	30 min.	18 hours	0.21	0.18	0.53	1.93
	Rabbits	Adequate	60 min.	1 hour	0.19	0.16	0.52	1.77
	Rabbits	Adequate	60 min.	18 hours	0.21	0.18	0.49	2.07
4	Monkeys	C-stored	Control	--	0.16	Not done	0.27	0.51
	Monkeys	C-stored	30 min.	1 hour	0.15	Not done	0.36	0.64

\*All experiments were done in June, 1938.

It will be seen from Table I that exposure of guinea pigs, rabbits, and monkeys to ultraviolet light for from thirty to sixty minutes caused no change whatsoever in the vitamin C content of the brain, liver, spleen, and adrenal gland. The average figures for the rayed and unrayed groups, respectively, were almost exactly identical, the range relatively small. This was true for guinea pigs which entered the experiment with partially depleted vitamin C concentrations as well as for guinea pigs which had been permitted to accumulate reserves by previous high level feeding.

The outcome of the above experiments was so clearly negative, irrespective of whether one was dealing with animals capable or incapable of synthesizing vitamin C, that there could be no doubt about the inefficacy of our method of irradiation in disturbing vitamin C metabolism. It was quite evident, however, that our rayed animals at no time exhibited any erythema on the skin despite the fact that the same lamp, even when operated at a greater distance, induced a very intense erythema on human skin after only three minutes' exposure. Marked irregularities in attempts to produce an erythema on the skin of guinea pigs by powerful ultraviolet irradiation have previously been reported by Ellinger.<sup>17</sup> The reason for this discrepancy between the reactivity of human and animal skin is not clear, and we have no explanation to offer for it. However, it occurred to us that the failure of the animals to develop a burn from the ultraviolet light might possibly account for the absence of vitamin C changes; conversely, if a burn were produced

by another more drastic method, such changes might ensue. We have, therefore, taken another group of guinea pigs, kept one-half for controls, and cauterized the shaven skin of the other half by the brief application of a searing knife over an area of approximately 2 cm. by 2 cm. The lesion thus provoked was entirely superficial, and, while not leading to any severe tissue destruction, proved sufficiently intense to cause discolorization of the epidermis with very slight scab formation. This experiment was carried out three times, once in summer and again in the winter and spring, in each case using duplicate animals, all of which had been kept before on an adequate diet. The results of the three vitamin C assays appear in Table II.

TABLE II  
EFFECT OF SKIN CAUTERIZATION ON VITAMIN C LEVELS OF TISSUES

ANIMAL	DIET	CAUTERIZATION	INTERVAL AFTER CAUTERIZATION	VITAMIN C CONTENT OF ORGANS (MILLIGRAM PER GRAM)			
				BRAIN	LIVER	SPLEEN	ADRENAL GLAND
Guinea pigs	Adequate	Control*	-----	0.15	0.16	0.38	1.04
Guinea pigs	Adequate	Cauterized	18 hours	0.13	0.07	0.15	0.38
Guinea pigs	Adequate	Control†	-----	0.11	0.12	0.25	0.51
Guinea pigs	Adequate	Cauterized	18 hours	0.09	0.06	0.18	0.35
Guinea pigs	Adequate	Control‡	-----	0.10	0.04	0.11	0.25
Guinea pigs	Adequate	Cauterized	18 hours	0.09	0.02	0.10	0.37

\*Experiment done in June, 1938.

†Experiment done in February, 1939.

‡Experiment done in April, 1939.

It is clear from the data given in Table II that the burned guinea pigs experienced in all organs save one a more or less pronounced drop in vitamin C levels; this drop reached its greatest magnitude in the liver. The change was well marked in all three experiments, although the influence of seasonal variation made itself felt in a tendency for the vitamin C values of all tissues to run noticeably higher in the summer than in the winter and spring. A similar phenomenon was observed before in our vitamin C assay of monkey tissues.<sup>16</sup> The levels found for normal control tissues compare favorably with the range reported in the literature for guinea pigs that have been kept on an adequate diet.<sup>18</sup>

#### DISCUSSION

The experimental data presented in this paper demonstrate that prolonged ultraviolet irradiation of albino guinea pigs, rabbits, and monkeys, fails to bring about any change in the tissue levels of vitamin C as it also proves singularly ineffective in inducing an erythema on the shaven skin of the rayed animals. However, when a superficial burn is produced on the skin of guinea pigs by the local application of heat, a more or less marked diminution of the vitamin C stores occurs in all the organs tested. The latter observation is in complete harmony with similar findings of Einhauser.<sup>13</sup> It is possible that toxic, histamine-like substances, which have an adverse effect on adrenal function, originate from the burned tissue and require large amounts of vitamin C for their detoxification,<sup>19</sup> a process which may be analogous to the well-known

depletion of vitamin C following the necrotizing action of diphtheria toxin. The fact that ultraviolet irradiation, without inducing visible tissue damage in laboratory animals, also leaves the vitamin C stores intact is of particular interest in view of Wright's<sup>10</sup> observation that evidence of disturbed vitamin C metabolism occurred only in one individual who showed definite intolerance to ultraviolet light exposure. Evidently, a mechanism operates in some individuals which protects tissues against damage and preserves the vitamin C reserves in the tissues; vice versa, the absence of such a protective mechanism in other individuals, or its breakdown by overdosage of light, may initiate a derangement of vitamin C metabolism. This, in turn, may assume significance as a contributing factor in the lowering of natural resistance against certain infectious diseases.

#### SUMMARY AND CONCLUSIONS

1. Exposure of guinea pigs, rabbits, and rhesus monkeys to prolonged ultraviolet irradiation caused no change in the tissue levels of vitamin C.
2. Such irradiation failed to produce any evidence of erythema on the skin of the rayed animals.
3. The production of a superficial burn on the skin of guinea pigs by the local application of heat was followed by a more or less marked drop in the content of vitamin C of the brain, liver, spleen, and adrenal gland.
4. Excessive doses of radiant energy apparently cause a derangement of vitamin C metabolism only to the extent that they are capable of producing injury to the tissues.

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## GONORRHEAL MYELITIS WITH ASSOCIATED PORPHYRINURIA FOLLOWING SULFANILAMIDE\*

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MYELITIS is one of the rarer complications of gonorrheal infection. Grinker<sup>1</sup> states, "Gonorrhea has been known in several instances to cause a myelitis. Apparently the organism ascends the urinary tract and either enters the nervous system by way of the venous channels or directly along the nerves. It produces an actual inflammatory destruction of the lower end of the spinal cord and gradually ascends." Blumer<sup>2</sup> says, "In patients with paraparesis following gonorrhea there is often great difficulty in deciding whether the lesion is a neuritis or myelitis. There is little doubt that true myelitis may occur." Gowers<sup>3</sup> gave a vivid clinical description of a rapidly progressive case of gonorrheal myelitis with fatal termination. Phifer and Forster<sup>4</sup> collected 29 cases from the literature to which they added 2 of their own. Gray<sup>5</sup> reported 5 cases of myelitis and encephalomyelitis with autopsy report in one. He stated that probably 70 to 80 cases of myelitis, meningo-myelitis, or encephalomyelitis have been reported in which the development was directly associated with gonorrhea. He found 64 cases in the literature. All but 3 were in males. In his own series 4 of the 5 patients were females. The autopsy report was on a female patient. The important findings were encephalomalacia limited to the fiber tracts of the internal capsule and the cerebellum, a few perivascular round cells, and unusual calcification of many of the smaller arterioles in the brain. He discussed the possibilities of direct infection, of associated virus infection or toxic manifestation. He thought the pathologic findings indicated a toxic effect being the most likely in the case studied by him. Search of available literature has disclosed very few additional reports. Marinesco, Dragenesco, and Chiser<sup>6</sup> discussed the development of a myelitic gonococcus vaccine reaction which is of some interest in connection with the case here reported.

The patient described below showed, along with the neurologic findings of myelitis, the presence of porphyrin pigments in the urine. Consequently, various laboratory studies were carried out along with clinical observation of the changes in the nervous system.

### CASE REPORT

A white male shoe worker, aged 26 years, was admitted to Our Saviour's Hospital, 11:30 P.M. on Nov. 5, 1937. His present illness began October 20 with urethral discharge, redness at the urinary meatus, urinary burning, and frequency. He consulted a physician two days later when the diagnosis of urethritis was made. He returned to his physician on October 25 and received a hypodermic injection. A report from the physician indicated

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gonococcus filtrate was given on October 25 and October 28. The patient stated he started sulfanilamide on the earlier date, taking four tablets four times a day for three or four days. These made him sick and dizzy so he cut the dose to one tablet four times a day for three days, then increased it to two four times a day until the day before entrance. This would give a total dosage of 460 to 540 grains of sulfanilamide over ten or eleven days, depending upon whether the initial dosage was kept up for three or four days, a point on which the patient was not certain. He felt chilly, dull, and draggy on October 31 and went to bed the following day. The urine was observed to be reddish in color after the injections with nocturia noticed somewhere in that period. This patient first noticed numbness of his feet on November 3, and became paralyzed the next day. He had increasing difficulty in urinating and required catheterization on the morning of November 4 and again on the day of entrance. Constipation had been increasing, but when castor oil was given the day before entrance, control of the bowels was lost and was not regained. On November 2 the patient had a chill and fever; on two days following he had two chills; on day of admission, none. He had vertigo since November 2. His vision was not clear on November 4 but returned the following day.

The past history of the patient was not remarkable. He was an inside worker and worked at his present occupation as a cutter of lining leathers in a shoe factory for eight years. He did not come in contact with dyes; the leather was already treated when he received it. His family history was negative.

His admission temperature was 102°, pulse rate 120, respiration 24. He was of slight build, rather undernourished and toxic appearing, conscious yet dull. He was pale with a suggestion of cyanosis. Pupils reacted promptly to light and accommodation. The fundi appeared normal. There was a toxic odor to his breath. No abnormal findings were noted in examination of his heart or lungs. Blood pressure was 100/60. There was tenderness in the suprapubic region where a distended bladder was found. There was a slight amount of thick yellowish urethral discharge with some redness at the meatus. Rectal examination was negative save for a relaxed sphincter. Extremities showed slight capillary cyanosis of the nails and edema of the ankles.

*Neurologic examination:* Complete anesthesia existed below the twelfth dorsal segment with a burning paresthesia just above it. There was some complaint of paresthesia in the hands. Muscle joint and vibration sensation were absent in the lower extremities which exhibited a flaccid paralysis from the waist down. The reflex findings were absent jaw jerk, upper deep equal but diminished, lower deep absent, epigastric, abdominal, cremasteric and plantars absent, Babinski present bilaterally with a definitely suggested bilateral Gordon and Oppenheim. There was no neck sign or Kernig.

*Laboratory studies: Urine.* On November 6 a catheterized specimen of deep red, clear, acid urine, with a specific gravity of 1.024 was obtained; it contained a trace of albumen but no sugar; microscopic study revealed a few pus cells. All urine was saved for various special examinations noted below. A slight trace of sugar was found on November 11 and from November 16 to 20; at all other times tests for sugar were negative. Albumen varied from negative to trace throughout the period of hospitalization. Granular casts were seen on November 11, November 15 to 18, and on November 28. Pus cells varied from negative to two plus at different times. Tests for blood, bile, hemoglobin, diazo reaction, and iodine, salicylic acid, phenacetin, rhubarb, senna, phenolphthalein, and pyramidon on the pigmented urine on November 6 were all negative. Sulfanilamide determinations, using the method of Marshall, Emerson, and Cutting,<sup>7</sup> gave the following results:

DATE	TIME	URINE VOLUME	SULFANILAMIDE (TOTAL)
November 6		720 c.c.	290 mg.
November 6	A.M.	480 c.c.	204 mg.
November 7	P.M.	960 c.c.	166 mg.
November 7	A.M.	960 c.c.	109 mg.
November 8	P.M.	960 c.c.	Too low to read



Spectroscopic examination of the urine tested by the simpler methods, as outlined by Mason, Courville, and Ziskind<sup>8</sup> and by Schumm<sup>9</sup> gave the following results:

DATE OF TEST	DATE OF URINE	METHOD	RESULT
November 8	November 6, A.M.	Garrod	Negative
November 8	November 7, A.M. and P.M.	Langnecker	Spectrum of acid hematoporphyrin Deep red fluorescence with ultra-violet light
November 9	November 6, P.M.	Garrod	Trace acid hematoporphyrin
November 9	November 7, P.M.	H. Fischer	Negative

The spectroscope used was a Spencer, students' type, the property of the Physics Department of Illinois College, with 60 watt frosted Mazda bulb at 24 inches and solution depth of 1 cm.

*Blood.* Blood counts made on November 6 showed hemoglobin 74 per cent (Dare), W. B. C. 7,800; R. B. C. 4,400,000, differential neutrophils 57, small lymphocytes 40, large lymphocytes 2, and basophiles 1. Daily counts were made until November 29, then weekly counts until patient was discharged. Red cell count dropped to 3,600,000 by November 11 but gradually mounted to 5,000,000 the day before discharge. The Kahn test on blood serum was negative.

On November 7 the blood sulfanilamide was too low to read. On November 8 the sugar was 67 mg., urea 17.7 mg., creatinine 1.5 mg., uric acid 4.6 mg. per cent, and icterus index 100 plus.

Spectroscopic examination on November 8 indicated the presence of methemoglobin in diluted serum; on November 9 it was present in diluted whole blood.

*Urethral smear* on November 8 was positive for gonococci.

*Spinal fluid:*

DATE	CELL COUNT	GLOBULIN	WASSERMANN	LANGE
November 6	316	2 plus	Negative	Negative
November 8	32	1 plus	Negative	Negative
December 4	5	Negative	Negative	Negative

Cells in the fluid of November 6 were mostly lymphocytes. No organisms were seen.

*Progress:* The patient showed some intermittent elevation of temperature until ten days before discharge. Neurologic changes gradually cleared. Superficial sensation was barely noted over the thighs on November 11, but absent elsewhere. Differentiation of heat and cold, pain and touch was possible by November 15. Awareness of light touch as a burning paresthesia was present by November 18 along with a return of muscle joint and vibration sensation. Clumsy motion of the right leg and slight movement of the left toes were also noted that day. Three days later the patient was able to void a small amount of urine. Redness of the skin over the sacrum to the left of the midline was noted that day. This broke down to a trophic ulcer by November 25 but was healing well by December 3. On this date the knee jerks were present but the ankle jerks were still absent. Sensation was good. Muscle power was fair, muscular atrophy pronounced. Examination on December 16 showed superficial and deep sensation normal, all reflexes but the cremasterics present, sphincter control good. The patient was lifted into a wheel chair that day, and with assistance sat up for Christmas dinner. He left the hospital January 4, 1938.

*Subsequent course.* Strength gradually returned as nutrition improved. He returned to his occupation June 1 and worked regularly ever since. Recent neurologic examination showed no abnormal findings.

#### DISCUSSION

Several factors entered into the consideration of etiology of the myelitis in this case. Toxic effects of sulfanilamide on the nervous system had been reported. Porphyrinuria is in many instances associated with lesions of

the central nervous system. Photosensitization and dermatitis following sulfanilamide led to association between sulfanilamide, porphyrinuria, and the cord lesion as a possibility. Evaluation of all factors, as well as the progress, brought about the conclusion that the condition was a gonorrheal myelitis with associated porphyrinuria following sulfanilamide.

Most of the effects of sulfanilamide on the central nervous system have been seen in experimental animals receiving doses proportionately much larger than those ever given clinically. Excitement, muscular weakness, ataxia, and signs of stimulation and depression of the central nervous system with spastic rigidity and hyperesthesia of twelve to twenty-four hours' duration were seen in dogs studied by Marshall, Emerson, and Cutting. Longcope<sup>10</sup> states, "Many patients experience malaise, anorexia, dizziness, somnolence, nausea, headache and are depressed. Vomiting is not uncommon. These are symptoms which have been observed in animals intoxicated by the drug and are dependent, no doubt, upon a direct action of the chemical." Such symptoms and signs are different from the ones in the patient studied here. Optic neuritis was seen by Bucy<sup>11</sup> in a patient who developed central scotoma for red and blue, and relative scotoma for white, following one tablet of sulfanilamide, although it had been given previously. As noted in the findings reported here, the patient said that vision was blurred the day before admission but that it had cleared up. Rather careful examination of the fundi showed no changes, but no perimetric readings were made.

The findings indicative of nervous system involvement usually associated with acute porphyrinuria are of motor nature. Peripheral neuritis and ascending paralysis of Landry's type are the most prominent clinical types. Mason, Courville, and Ziskind's monograph gives a complete clinical and pathologic description. Boulin, Garcin, Nepveux, and Ortolan<sup>12</sup> reported acute porphyrinuria with peripheral paralysis showing pathologic changes in the anterior root cells in the cervical and lumbar areas in the cord. There were no sensory symptoms in their patient. Waldenstrom's<sup>13</sup> monograph is a most complete summary of clinical, chemical, and pathologic findings. He studied 50 patients in only one of whom were there sensory changes. He states (loc. cit. p. 103): "Sensation is in great majority of cases entirely intact. Only by long presence of symptoms have I been able to observe undoubted appearance of superficial as well as deep disturbance of sensibility. . . . As a rule only the motor neuron is involved." In the case studied sensory symptoms were among the first complaints in the numbness in the feet. Objective evidence of loss of superficial and deep sensation below the twelfth dorsal segment and description of paresthesia in the hands were definite.

When the first reports on photosensitivity following treatment with sulfanilamide appeared, the thought occurred of disturbance of pigment metabolism. A case of meningitis treated with sulfanilamide and meningococcus antitoxin under observation concurrently with the case studied also showed porphyrinuria. Burnsting's<sup>14</sup> report spoke of porphyrinuria following sulfanilamide. Rimington and Hemmings<sup>15</sup> clinical and chemical study showed that patients receiving sulfanilamide showed hematoporphyrinuria in amounts

nearly ten times that normally found, and that this continued even after the drug was stopped. The tabulation of clinical diagnoses in their article does not mention any central nervous system disorder.

Whether the use of gonococcic filtrate in the case studied was of any etiologic significance is quite speculative. A situation perhaps indirectly comparable is in the article by Marinesco, Dragenesco, and Chiser (*loc. cit.*) in which they described a myelitis of the herpes zoster type developing after seven injections of gonococcus vaccine. Their patient, however, had had poliomyelitis in boyhood and encephalitis in adult life and hence already had a damaged central nervous system. Gonococcus filtrate has been widely employed with many favorable and some unfavorable results reported. No central nervous system reactions have been seen described.

#### SUMMARY

A case of myelitis in a patient with gonorrhea has been described. The patient had taken sulfanilamide. The color of the urine suggested porphyrinuria which was confirmed by spectroscopic examination. Some of the possibilities of causation of the myelitis have been discussed with the conclusion that the diagnosis was gonorrheal myelitis with associated porphyrinuria following sulfanilamide.

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# THE HEMATOLOGIC STUDY OF 76 PNEUMONIA CASES TREATED WITH SULFAPYRIDINE, INCLUDING A FATAL CASE OF AGRANULOCYTOSIS\*

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THE introduction of chemotherapy in the treatment of pneumonia and other medical conditions naturally presents the problem of whether the drug in question has any toxic or other untoward effects. The use of sulfapyridine, M and B 693, or Dagenan, in the treatment of pneumonia was begun at the Toronto Western Hospital in October, 1938. At that time there was no literature concerning possible toxic effects of the drug upon the hematopoietic system, and we undertook this study. In the meantime, several reports on the use of sulfapyridine in the treatment of pneumonia and other infections have been published. The volume of this work is rapidly increasing, and a more complete knowledge of the biochemical reactions, clinical uses, and toxic effects of this chemotherapeutic agent is becoming available. No attempt has been made to present an exhaustive review of the literature of this subject, because the rapidity with which new knowledge of the drug is being published would quickly out-date any summary of this type.

## METHOD

Leucocyte and erythrocyte counts and hemoglobin estimations were taken before the institution of sulfapyridine therapy. Occasionally this procedure was not possible, and the work was started a few hours after the first dose was given. These estimations were repeated daily for the duration of the therapy and every two to three days thereafter until the patient's discharge. From time to time, where indicated, blood films were taken and examined with special attention to the differential leucocyte count. The estimations of the red and white blood cells were done in the usual manner. The hemoglobin readings were made using the standard Sahli instrument, allowing thirty minutes for the completion of the acid hematin reaction and correcting the readings to a standard of 15.4 gm. hemoglobin for 100 per cent. Frequent duplicate estimations of leucocyte, erythrocyte, and hemoglobin levels were made by two workers in order to assure accuracy of results.

## RESULTS

The series here recorded consists of all persons given sulfapyridine therapy after the scheme of investigation outlined above was established, and comprises 70 patients. Six of these had two separate courses of the drug because of a

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second attack of pneumonia, extension, empyema, etc. The hematologic record of these patients was followed with each course of therapy and is included in our results as separate cases. The pharmacologic and clinical aspects of the whole series of cases have been recorded by Brown, Thornton, and Wilson,<sup>1</sup> and Detweiler, Kinsey, Brown, and Feasby,<sup>2</sup> and submitted for publication separately.

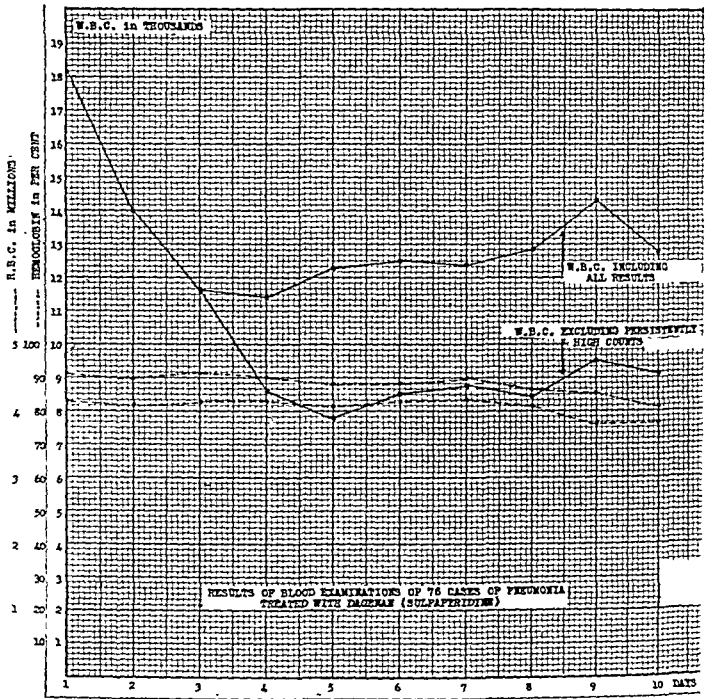


Chart 1.

In Chart 1 are recorded the results of the tabulation of blood examinations from the first to tenth day inclusive, showing the average levels of leucocyte, erythrocyte, and hemoglobin estimations. Study of this graph revealed a leucocyte fall during the first four days, with a slight elevation beginning on the fifth day and lasting to the tenth. The persistence of a leucocytosis of 12,000 or over occurred in 35, or nearly half, of the persons studied. The rest had levels within the normal range, as shown in the chart, throughout the remainder of their stay in the hospital. To determine the possible significance of the persistent leucocytosis, the 35 persons were separated for further analysis. Twelve of them showed no cause for leucocytosis, though 2 had had positive blood cultures, 2 had oliguria, 2 hematuria, and one had diabetes. The leucocyte levels, however, were not of a high nature, the maximum being 22,300. The remaining 23 patients showed a variety of complications and concomitant disease states as follows: simple empyema 4, simple effusion 2, effusion and pneumonic extension 1, effusion and delayed resolution 1, effusion and peripheral phlebitis 1, delayed resolution 2, pneumonic extension 1, extension and infective asthma 1, and pneumonic extension and empyema 1. The remainder of this group showed the following associated conditions: infective asthma, severe pulmonary tuber-

culosis, gonococcal arthritis, salpingitis, postgastroenterostomy and lung abscess, postcholecystectomy, carcinoma of breast, and ureteral calculus with probable kidney infection.

Though this series is admittedly small in number, it is thought to be of significant importance to stress the fact that of 35 patients with a persistently high leucocyte level after the third day of sulfapyridine therapy, 65 per cent presented a definite complicating factor, and of these, 46 per cent were of a pulmonary nature.

Examination of the average erythrocyte and hemoglobin levels showed, over the ten-day period, a decline of the former from 4,580,000 to 4,010,000, and of the latter, from 82.6 per cent to 76.6 per cent. Even without considering the factors of fever and dehydration, which produced elevated blood levels in the first two to three days, the total drop in erythrocyte and hemoglobin levels was not beyond what is anticipated in this type of acute infective processes. The maximum fall of hemoglobin occurred in a 16-year-old boy who developed effusion and empyema, and whose hemoglobin fell from 90 to 63 per cent in a nine-day period. The maximum fall of from 5,700,000 to 4,100,000 erythrocytes in ten days occurred in a 21-year-old man who also developed empyema. In these cases, fever and dehydration probably produced the initial elevated level of erythrocyte and hemoglobin estimations. Furthermore, in each of these cases the causative agent was a hemolytic streptococcus.

Apart from the foregoing results there were no hematologic observations which would indicate any tendency for the drug in question to cause a hemolytic type of anemia. One patient, J. R., recorded in detail in this study, received a dosage of 85 gm. of the drug over a period of 16.5 days, with no hemolysis occurring. Another patient receiving high dosage without developing anemia was H. F., a male, who was given 48 gm., in seven days, with an average daily intake of 6.9 gm. This patient had a short period of anuria during which the blood levels reached 32.6 mg. total and 16.6 mg. per 100 c.c. free drug.

Leucopenia was considered to be present when the leucocyte count fell to a level below 5,000. Of the 26 cases in this series, depression of the leucocytes below this number occurred in 8 patients in whom 27 low estimations were recorded. In only 2 cases was the leucocyte depression noted but once, while the most persistent leucopenia observed was one in which the condition was present on seven successive days. Blood-film examinations presented differential counts which varied with the degree of depression. When the total count was between four and five thousand cells, the lymphoid elements were relatively increased, but retained their absolute number. With a further decline of the leucocyte level, there was an absolute fall of both lymphoid and myeloid elements, with maintenance of the relative lymphocytosis. Detailed examination of the white blood cells revealed no significant changes in the lymphocytic or monocytic elements. The neutrophils presented little change beyond a moderate shift to the left and an increase in toxic granularity. There were no forms seen which were less mature than the juvenile type. The erythrocytes revealed but little variation from normal, the only change noted being the appearance of an occasional polychromatic cell. Table I presents the most significant features of the patients who developed a leucopenic state.

TABLE I

PA-TIENT	AGE	SEX	LEUCOCYTE COUNTS		DAGENAN DOSAGE (GM.)			HIGHEST NOTED DAG-ENAN BLOOD LEVELS (MG. PER 100 C.C.)			CAUSATIVE ORGANISM
			AD-MIS-SION	LOW-EST	TOTAL	DAYS GIVEN	AV. DAILY DOSE	TOTAL	FREE	CONJ.	
T. W.	16	M	23,000	4,500	6.0*	1.0	6.0	6.9	6.7	0.2	Pneumococcus type II
M. B.	14	F	5,400	2,600	10.0	1.0	10.0		6.4		Pneumococcus†
V. B.	41	F	8,500	3,000	42.0	7.0	6.0	11.8	7.8	4.0	Pneumococcus†
B. M.	50	F	6,100	3,400	14.0	2.0	7.0		9.3		Pneumococcus type I
J. S.	47	M	18,600	4,500	22.5	3.5	6.3	17.6	14.4	3.2	Pneumococcus type IV
F. L.	32	F	25,400	2,800	30.0	6.0	5.0	5.4	3.4	2.0	Pneumococcus type VII
J. C.	27	M	5,300	3,300	16.0	2.5	6.4	10.4	9.5	0.9	Pneumococcus type VI
J. R.	59	M	7,700	700	85.0	16.5	5.1	26.3	13.8	12.5	Hemolytic streptococcus
Average			11,250	3,100	28.2	4.9	6.4	13.0	8.9	3.8	
Total Series Average					33.8	6.5	6.2	13.0	9.9	3.0	

\*Intravenous soludagenan (sodium sulfapyridine) 2 doses of 3 gm. each.

†Pneumococcus did not type.

The histories of all 8 patients were carefully rechecked, and it was found that they all had typical signs and symptoms of pneumonia, with confirmatory x-ray examinations in several cases. The age range was from 16 to 59, and the cases were equally divided between the sexes. The initial leucocyte counts were low, only 3 being above 8,500. The totals of sulfapyridine dosage and the number of days the drug was given are lower than the average of the whole series, because with the onset of leucopenia, the drug was discontinued. The highest levels of drug concentration in the blood are comparable to those of the series as a whole, but were not maintained because of discontinuance of the drug. There was no predominating type of pneumococcus. In the case of the sputa which did not type, sulfapyridine had been given before the sputum was obtained for typing.

*Agranulocytosis.*—At the time of presenting this study, the medical literature contains reports of at least 4 cases of agranulocytosis following the use of sulfapyridine. These include the cases of Barnett and co-workers,<sup>3</sup> Johnston,<sup>4</sup> Coxon and Forbes,<sup>5</sup> and Sutherland.<sup>6</sup> We have had one case. All of these cases, as well as our own, received large doses of the drug, as shown in Table II.

TABLE II

CASE	DOSAGE IN GM.	DAYS GIVEN	AV. DAILY DOSE	ORGANISM
Barnett et al.	80.9	17.0	4.8	Pneumococcus type I
Johnston	54.0	11.0	4.9	Streptococcus viridans
Coxon and Forbes	54.0	17.0	3.2	B. typhosis
Sutherland	52.0	12.5	4.2	Streptococcus hemolyticus
Morgan	85.0	16.5	5.1	Streptococcus hemolyticus

In addition to the foregoing incidences of agranulocytosis, Tzanek and associates<sup>7</sup> report a case of agranulocytosis where only moderate doses of the

drug were employed, and we have knowledge of a further fatal case which occurred in another Toronto hospital. The most important features of our case of agranulocytosis are included in the following case report.

J. R., a male, aged 59 years, was admitted to the medical division March 9, 1939. He gave a history of asthma of two years' duration, and weakness, malaise, headache, and slight cough for one week. Examination revealed an acutely ill patient who was dyspneic and slightly cyanosed. Physical and x-ray examinations revealed pneumonia of the right upper lobe. The patient was given sulfapyridine therapy in the dosage recorded in Chart 2. The temperature fell to normal, and after six days' treatment, the sulfapyridine was discontinued. The following day the fever returned, and the use of the drug was resumed. During this course of the drug the patient developed a maculopapillary rash and oliguria, as noted on the chart.

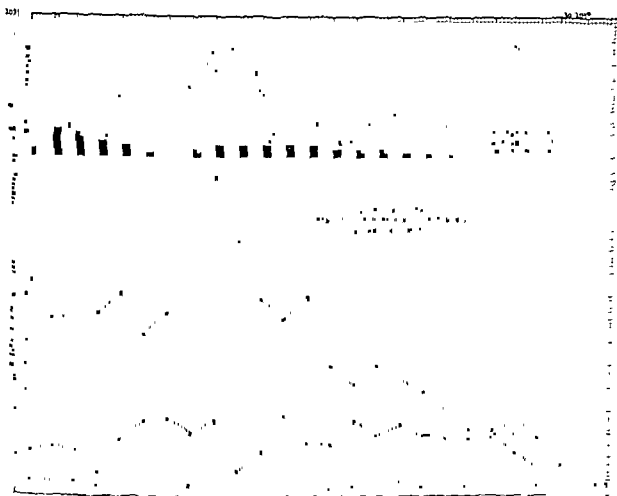


Chart 2.

After twelve days he had a normal temperature, felt well, and had no abnormal pulmonary findings, and the drug was discontinued. Two days later his fever again returned, and he had evidence of pneumonia in his left lower lobe. At this time his leucocytes numbered only 2,200, with 100 per cent lymphocytes. He was given repeated transfusions and daily intramuscular liver injections, but his leucocyte level continued to fall, and in three days his count was only 700. The pneumonic process spread to all lobes, and he died the twenty-third day after admission.

Bacteriologic examination revealed *Streptococcus hemolyticus* with good inhibition of growth in vitro in 5 and 10 mg. per cent sulfapyridine in broth. The essential features of the autopsy were as follows: Both pleura showed old dense adhesions over all surfaces, with fresh inflammatory reaction evident over the lower lobes; there were 200 c.c. of blood-stained fluid in the left pleural cavity. Macroscopically both lungs revealed a generalized pneumonic process in various stages. Microscopically the lung tissue, due to the absence of granulocytes, presented the appearance of a pulmonary edema rather than a pneumonic process. Marrow, removed by sternal puncture immediately after death and from the femur and ribs at autopsy, showed a complete lack of granular cells. Several myeloblasts were present, but no evidence



of any maturation of these cells was found. There did not appear to be any change in the character of the erythrocytic series or arrest in their development. The thrombocytes were unaltered.

On admission the leucocyte count of the patient was only 8,700, and during his illness only one count was obtained above 9,000. There was no significant decline in hemoglobin or erythrocyte levels. He developed a rash ten days before his agranulocytosis. The total dosage of 85 gm. was given over a 16.5 day period, with an average of 5.1 gm. daily. The highest blood levels reached were 26.3 mg. per 100 per cent total, 13.8 free, and 12.5 conjugated. At this time he had a high level of the conjugated form, which is thought by some workers to be more toxic than the free. This phase of the series is fully covered in a paper by Brown, Thornton, and Wilson,<sup>1</sup> which is now in the process of publication. This patient received the second greatest total amount of the drug of any of this series, though his average daily intake was not as high as the average for the whole group. While his blood levels were quite high initially when the doses were larger, his later figures were comparatively low.

The small series of agranulocytosis here presented shows that large doses were given over a comparatively long period of time before onset of the fatal condition. Without attempting to present a complete analysis of these cases, it seems apparent that the type, virulence, and extent of bacterial infection are the factors which determine the high dosage required to provide adequate chemotherapy. That the drug may have definite toxic effects on the development of the granular leucocytes is obvious. The onset of leucopenia, as previously shown, is not of necessity related to the intake of the drug, but it seems that agranulocytosis may develop with doses above 50 gm. As further reports are published, it is probable that this condition will be found to develop with smaller total doses, since it appears that there must be, as revealed by our series of leucopenic cases, an individual susceptibility of certain patients to the toxic effects of the drug. It is hoped that with the accumulation of data, more definite knowledge of the toxic properties of sulfapyridine may be obtained. Until an improved method of determining deleterious effects on the neutrophile series is found, it is considered necessary to do daily leucocyte counts and discontinue the drug immediately upon the appearance of leucopenia.

#### SUMMARY

The number of cases included in this study is admittedly small, and it is probably inopportune to attempt to draw any concrete conclusions from a group of this size. It is hoped, however, that our results, taken in conjunction with those of other workers, will assist in revealing the possible toxic effects of the drug upon the hematopoietic system.

Of 35 patients with a persistent leucocytosis after the third day of sulfapyridine therapy, 65 per cent had a demonstrable complicating factor, and of these, 46 per cent were pulmonary in character.

Sulfapyridine did not produce any significant decline in hemoglobin or erythrocyte levels.

Leucopenia developed in 8 out of 76 cases, approximately 10 per cent. There was a relative lymphocytosis when the total leucocyte count fell to 4,000; below this level the cells of both the lymphocytic and granular series were reduced in total number, with the relative lymphocytosis being maintained. There were no significant changes in cell characteristics.

The leucopenia did not seem to be related to age, sex, causative organism, dosage, or blood levels of the drug. It appears evident that patients with low initial leucocyte counts are much more liable to leucopenia.

A case of sulfapyridine-treated hemolytic streptococcal pneumonia, which developed a fatal agranulocytosis, is reported in detail. It seems evident that, until more complete knowledge of the drug is obtained, daily hematologic examinations are necessary so that with the development of leucopenia, the drug may be immediately discontinued before agranulocytosis may supervene.

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#### A STABLE ADRENAL CORTICAL EXTRACT\*

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ADRENAL cortical extracts have been prepared by various workers.<sup>1-4</sup> When freshly prepared, the extracts of Hartman and Swingle and Pfiffner were found successful in prolonging the life of adrenalectomized animals by various workers,<sup>5-7</sup> but the potency of these extracts is rapidly diminished even at icebox temperatures, so that by the end of two to three weeks these extracts are found to be ineffective. More recently, Cartland and Kuizenga<sup>8</sup> reported a method of preparing adrenal cortical extracts which they claim remain stable for longer periods.

This paper describes a method of preparing an adrenal cortical extract, which tends to retain its potency upon standing at room temperature for at least eighteen months.

#### METHOD

Whole adrenal glands from freshly slaughtered cattle, collected on the floor of the abattoir, are chilled in carbon dioxide ice and immediately brought to the laboratory where they are finely ground in a meat chopper. Each pound

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of gland is placed in one liter of 95 per cent alcohol and shaken in a mechanical shaker from sixteen to twenty hours, as originally described by Hartman.<sup>9</sup> The glands are then pressed in an ordinary wine press through a dish towel. The residue is discarded, though it may, however, be re-extracted with 80 per cent alcohol, using one liter per pound of gland, and again shaking as described above. The second extraction adds 10 to 15 per cent to the potency of the final extract. The alcoholic extracts are filtered and evaporated in vacuo at 45° C. (The insertion of a capillary tube, provided with a rubber tubing and a screw clamp into the rubber stopper, effectually prevents foaming during evaporation and also oxidizes the adrenalin. By adjusting the screw clamp, enough air is admitted to break up the films and yet maintain sufficient negative pressure to insure rapid distillation.) The aqueous sludge, which remains after the alcohol is evaporated, is washed with equal volumes of benzol until the last washing of benzol is colorless. Usually four to six washings are sufficient. The aqueous sludge is discarded. The benzol portion is evaporated in vacuo to a thick paste. The residue is dissolved in 70 per cent alcohol, using 100 c.c. of alcohol for each pound of original gland. The alcoholic extract is placed for at least twelve hours in an icebox 4 to 6° C. A heavy, dark precipitate is brought down, which can be separated by filtering in the icebox. This precipitate, which melts at room temperature, is very toxic to dogs and rats. The alcoholic filtrate is then evaporated in vacuo, and when the alcohol has evaporated, the aqueous portion is again washed with equal volumes of benzol until the benzol is colorless. Usually three to five washings are sufficient. The aqueous portion is discarded. The benzol is evaporated to dryness, and the residue is dissolved in 70 per cent alcohol, using 50 c.c. of alcohol for each pound of original gland; the alcohol is again placed overnight in the icebox. The process of washing the aqueous portion with benzol, and redissolving in 70 per cent alcohol and placing in the icebox as just described is repeated until no more precipitate is formed. When the precipitate is no longer formed from standing overnight in the icebox, the aqueous portion is washed with benzol, which in turn is evaporated to dryness. The residue is dissolved in 95 per cent alcohol (10 c.c. per pound of original gland) and this again is evaporated to dryness. The residue is extracted in 20 per cent alcohol for thirty to forty minutes. The amount of 20 per cent alcohol used in the last extraction may be varied according to the concentration one desires. The final extraction is passed through a Seitz filter. (The plates of the Seitz filter are alkaline, pH 10 to 11; therefore, it is best to wash the plates with 100 c.c. of 6 per cent acetic acid, and wash the acid out with 400 c.c. of water.) The final extract has a clear light yellow color.

The 20 per cent alcoholic extract is miscible with any volume of water or alcohol. In this work, enough alcohol was added to make it 50 per cent by volume and kept at room temperature from one to eighteen months. Before using the 50 per cent alcoholic extract, the extract was diluted to a concentration of 10 per cent alcohol; however, when the 20 per cent alcoholic extract is used, it may be injected without dilution.

The final extraction may be made with water, but in the aqueous state the extract is not stable.

#### CHEMICAL ANALYSIS

No detailed and exhaustive chemical analyses have been made on the extract.

The extract is free from nitrogen. The adrenalin content must be very low, for 5 c.c. of extract injected intravenously has no effect on the blood pressure of cats anesthetized with ether.

Eagle,<sup>10</sup> who has found considerable quantities of choline in adrenal cortical extracts prepared according to methods of other workers, found 1.8 and 1.2 gamma per c.c. of choline in two samples of this extract.

#### METHOD OF ASSAYING

Dogs, apparently normal, were adrenalectomized in two stages. The animals were kept on the same diet before and after surgery. The diet consisted of ground meat mixed with bread and bone meal (the standard diet used in this laboratory). The dogs were fed once a day. Water was always present in their cages.

Subcutaneous injections of extract were started on the second day after the second adrenal gland was removed. It was found that dogs survived better under this regime than they did when the extract was started when definite symptoms of adrenal insufficiency were in evidence. The injections of the extract were continued ten to twelve days and then withdrawn. When symptoms of adrenal insufficiency set in, the administration of the extract was resumed and continued until the animal appeared normal. Again the extract was withdrawn. When the dog showed signs of adrenal insufficiency, injections of the extract were again resumed and continued until the animal was apparently normal. Final withdrawal of extract led to speedy exitus.

A necropsy was performed on each dog. Macroscopic examination never revealed any remains of adrenal cortical tissue.

An effective dose was considered one which would keep the animal not only alive, but obviously in good physical condition. It was found that not every batch of glands extracted yielded the same potency on the same dog; nor were the requirements of each dog per kilogram of body weight the same. For example, Dog 30, weighing 9.4 kg., required 5 c.c. of extract 128, while Dog 4, weighing 12 kg., required 10 c.c. of the same extract to keep it in good condition. Using extract 131, Dog 30 required 10 c.c. per day to keep it in good condition, in contrast to 5 c.c. of extract 128. Both extracts represented the same amount of whole adrenal gland per cubic centimeter of extract, namely, 45 gm. of fresh gland.

In all, 29 dogs were used in this work, 10 of which were used as controls to note the survival period of bilaterally adrenalectomized dogs without therapy, and 19 were treated with extract. The survival period of the control dogs was, on the average, four days. The longest survival period was five days, and the shortest, two days. The treated animals were permitted

to live on the average for twenty-three days. The longest period a treated dog was permitted to live was thirty-two days, and the shortest period, twenty-one days. In this group, each dog had adrenal insufficiency at least twice and recovered with extract therapy. Final withdrawal of extract caused death within four days.

Following is a protocol of a typical experiment.

- Dec. 21, Removed right adrenal.
- Feb. 9, Removed left adrenal.
- Feb. 11, Dog did not stand up and did not eat. Injected 10 c.c. extract 107B.
- Feb. 12, Dog stood up and ate its food. Injected 10 c.c. of 107B.
- Feb. 13, Dog was in fine condition. Injected 8 c.c. of 107B.
- Feb. 14, Dog was in fine condition; ran around and was very active. Injected 8 c.c. of 107B.
- Feb. 15, Dog was in fine condition. Injected 12 c.c. of 107B2.
- Feb. 16, Dog was in fine condition. Injected 12 c.c. of 128.
- Feb. 17, Dog was in fine condition. Injected 10 c.c. of 128.
- Feb. 18, Dog was in fine condition. Injected 6 c.c. of 128.
- Feb. 19, Dog was in fair condition. It was not as active as on previous days. Injected 10 c.c. of 128.
- Feb. 20, Dog was in good condition, and was again very active. Injected 10 c.c. of 128.
- Feb. 21, Dog was in fine condition. Injected 10 c.c. of 128. This dog was among other dogs and a fight ensued in which this dog also participated. In the evening it was very weak. Injected an additional 10 c.c. of 128.
- Feb. 22, Dog was able to run around and appeared to be in good condition. Injected 6 c.c. of 128.
- Feb. 23, Dog was in fair condition. No extract administered.
- Feb. 24, Dog was in fairly good condition, did not eat all of its food, and vomited. No extract was administered.
- Feb. 25, In the morning the dog was able to walk around. In the evening its gait was staggering and it was unable to climb into its cage. No extract was administered.
- Feb. 26, At 6 A.M. the dog was unable to stand up; it vomited the little food it had eaten. Injected 15 c.c. of extract 128. At 12:40 P.M. the dog was able to rise and walk a little. Injected an additional 5 c.c. of 128. At 8 P.M. the dog was able to run around, appeared to be in excellent condition, and ate its food.
- Feb. 27, Dog was in excellent condition. Injected 5 c.c. of 128.
- Feb. 28, In the morning the dog was in excellent condition, but in the evening it was very lethargic. No extract was administered.
- March 1, Dog had a staggering gait so typical of adrenal insufficiency. Injected 9 c.c. of extract 109BHO.
- March 2, Dog was in excellent condition. No extract was administered.
- March 3, Dog was lethargic and refused to eat. No extract was administered.
- March 4, In the morning the dog was unable to walk; it was very unresponsive all day; in the evening it had convulsions.
- March 5, Dog died in the morning.

At necropsy no visible adrenal cortical tissue was found. The gut was hyperemic, but otherwise there were no visible changes.

#### DISCUSSION

The method here outlined differs essentially from other methods of preparing adrenal cortical extract, by the use of only two extractives, alcohol and benzol (both being relatively inexpensive). The laborious method of removing adrenalin by adsorption to permutit of Swingle and Pfiffner<sup>11</sup> is eliminated. The shaking during the extraction and the admission of air during the evaporation oxidizes the adrenalin.

Almost any lipid solvent may be substituted for the alcohol and benzol; there is no advantage as far as potency is concerned, and the cost of most other solvents, such as acetone, chloroform, and ether, is greater. Although the final extraction is made with alcohol, the potent alcoholic extract can be diluted with water or physiologic saline in all proportions; thus, one may avoid any damage to tissues following injections. Repeated subcutaneous injections of 20 per cent alcohol in dogs caused no demonstrable deleterious effects.

Although other workers<sup>1-7</sup> kept adrenalectomized dogs and cats alive for longer periods than we did, the repeated symptoms of adrenal insufficiency which occurred when the extract was withdrawn and the recovery upon resumption of administration of extract show that the extract is able to keep adrenalectomized animals alive. There is no reason to doubt that dogs would not have lived indefinitely were the administration of extract continued.

#### SUMMARY

A new method of preparing adrenal cortical extract, which is stable for at least eighteen months and free from protein, is described.

I herewith wish to thank Dr. Carlson for his constant help and pertinent advice throughout this work.

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## PERSISTENCE OF INFECTION WITH *GIARDIA INTESTINALIS* IN MAN\*

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**H**UMAN infections with the intestinal flagellate, *Giardia intestinalis*, are of somewhat common occurrence, and are occasionally observed under circumstances which strongly suggest that the parasite is responsible for disease in the small intestine or gall bladder. As yet, however, the causal relationship of *Giardia* to either pathology or symptoms in man has not been established. Often the parasite is difficult to eliminate from one who harbors it, and infections may persist in the presence or absence of symptoms for long periods. Such a case of prolonged infection, with occasionally distressing symptoms, has come to our attention, and, in the present paper, we shall present some significant points of the history and other facts concerning it.

*History.*—When the infection was first determined, the patient was a student in a parasitology class at the University of California, Los Angeles. She was suffering from a severe diarrhea during March of that year (1934) and, on examining her own stool, observed both motile forms and cysts of *Giardia intestinalis*. Her observations and identifications were corroborated by the instructor of the class, Dr. Gordon Ball. About eighteen months later, during the summer of 1936, the patient suffered a very distressing intestinal upset lasting six weeks, with blood appearing in the diarrheic stools. In spite of careful study and search by the attendant physician, no specific cause for the symptoms could be found, although *Giardia* was again proved to be present. Carbarsone was administered as treatment to eliminate the protozoan, and, although the number of parasites was temporarily reduced thereafter, the forms never entirely disappeared and soon became as numerous as they had been originally. Eventually the intestinal condition improved, although no specific reason for the improvement could be ascertained. A few months later the patient moved to New York City, and in February, 1937, we found both trophozoites and cysts of *Giardia*. In January, 1938, and again in February, 1939, we again observed the cysts of the parasite in the formed stools of the patient. They were numerous, and unquestionably cysts of *Giardia*, as judged by examination of both a fresh saline emulsion of the feces and an emulsion to which iodine had been added.

*Symptoms.*—The patient occasionally suffers from intestinal disturbances, with more or less severe diarrhea and epigastric pain before breakfast or between meals during the day. The attacks are most intense in the spring and summer months. Sometimes blood appears in the stools. At such times, motile specimens of *Giardia* can be observed in the stools, although between such attacks, *Giardia* cysts alone have been found. The motile forms can be brought forth at will, nevertheless, by the administration of a purgative.

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## COMMENT

It is neither assumed nor suggested that *Giardia intestinalis* is the cause of the symptoms described in the case presented. Wenyon has pointed out that "blood never occurs in pure *Giardia* infections," and blood was found in the stools of this patient during the periods of intense diarrhea. Although no other agent which might cause this condition has been determined, we, nevertheless, leave open the question of the causal relationship of the parasite. This case has been reported only because of the long persistence of proved infection with the parasite, *Giardia intestinalis*.

It is not definitely known whether the patient represents a single prolonged infection or a succession of reinfections, although there seems greater likelihood for the former. This is indicated not only by the fact that the patient is of the upper social strata and lives under good hygienic and sanitary conditions, but also by the infection persisting after a major geographic shift—from the West to the East Coast. If it is true that this case does represent a single prolonged infection, then there appears to be a distinct difference between the duration of infection in this case with *Giardia intestinalis* and the infection of rats with *Giardia muris*. Hegner has demonstrated experimentally that rats lose their *Giardia* infections in about five months if reinfection is prevented. In his experiments, 20 rats kept in cages with a screen bottom so that fecal pellets fell through were free of their original infection in about five months. Nineteen of 20 control rats kept in a cage with shavings were found still infected after this interval. In contrast with this result in rats, the present case has harbored the human parasite for over five years and shows great promise of continuing to do so for an indefinite period.

## SUMMARY

A case is reported of prolonged infection of a patient with *Giardia intestinalis*, the parasite having been observed in the stools at intervals over a period of five years. It is noted that the persistence of this parasite in man may be distinctly greater than that of a related form, *Giardia muris*, in rats, since rats are found experimentally to lose their infection within five months if reinfection is prevented.

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# LABORATORY METHODS

## THE DETERMINATION OF UREA IN BLOOD AND URINE BY CONWAY UNITS\*

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SELJESKOG and Cavett<sup>1</sup> described the methods commonly used for the determination of urea in blood and compared them as to accuracy and simplicity in the November, 1937, issue of this JOURNAL. Although most of these methods gave efficient results, I would like to draw attention to a method which may be called the "Columbus egg" of the methods for the determination of urea. It is the modified method of Conway.<sup>2</sup>

For analyses on a large scale the Conway method has many advantages over other methods: (1) Only 0.2 c.c. blood or plasma are required for one estimation. (2) This method is the most accurate of all methods and is about twice as exact as Van Slyke's manometric procedure B.<sup>3</sup> If 0.5 c.c. blood are used, even Kirk's<sup>4</sup> very exact results can be exceeded in accuracy, and it is possible to determine the blood urea concentration with an accuracy of about 0.07 mg. per cent. (3) A large number of determinations can conveniently be accomplished simultaneously, the average time taken for one estimation being about four to six minutes. By suitable arrangement the action of the urease causes no loss of time at all. The diffusion proceeds without any supervision. (4) By Abelin's<sup>5</sup> modification the standard mineral acid for the reception of the ammonia is replaced by boric acid, and the ammonium borate is titrated directly with a standard mineral acid (viz., Brecher<sup>6</sup>). The standard alkali solution is replaced thus by the stable standard acid. The relatively strong boric acid in the receiver absorbs also abnormally high amounts of ammonia and, therefore, a special procedure for high urea values is unnecessary. For the same reason the estimation of urea in urine can be made with the same solutions, and the analyses of blood and urine can be performed simultaneously. (5) If only the U:P ratio and not the absolute urea values are required, the titration can be made with a nonstandardized acid, the U:P ratio being calculated by simple division of the titration values. (6) This method can be learned easily and makes clean and quiet working possible. The expense involved by the method is limited to not much more than the purchase of the Conway units, the cost of the reagents being very modest.

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It is obvious that a method with these advantages of accuracy and velocity is very suitable for scientific work and for everyday laboratory practice. In our clinic we have used this method for two years with the most satisfactory results for the determination of the urea clearance, and have saved much time in so doing. The introduction of the boric acid as an absorbing acid is a further step in the simplification of the method.

#### PRINCIPLE

The principle of the Conway unit\* consists in the diffusion of the ammonia formed from urea and liberated in the outer chamber of the very simple apparatus into the boric acid absorption solution in the inner chamber. Under the prescribed conditions the absorption is completed within one hour at 38° C. The ammonium borate is directly titrated with sulfuric or hydrochloric acid with a Conway microburette.<sup>8</sup> For the exact measures and figures of these apparatus see Conway's original papers.<sup>2, 6</sup>

#### REAGENTS

1. The fixative for the glass lid: 40 to 50 gm. solid paraffin are melted with 80 c.c. liquid paraffin.

2. One per cent boric acid in distilled water.†

3. Mixed indicator: 1 part 0.2 per cent methyl red in alcohol and 1 part 0.1 per cent methylene blue in alcohol.

4. Urease solution: Per unit 20 mg. highly active urease (Arleo urease) are dissolved in 0.5 c.c. water and 0.02 c.c. of the phosphate solution is added. The amount required is freshly prepared every day. In our practice the urease solution in 50 per cent glycerol (Sabine<sup>10</sup>) showed ammonia formation after some days. The phosphate solution contains 6.9 gm.  $\text{NaH}_2\text{PO}_4 + 2\text{H}_2\text{O}$  and 17.9 gm.  $\text{Na}_2\text{HPO}_4 + 12\text{H}_2\text{O}$ .

5. A saturated potassium carbonate solution is boiled vigorously for twenty minutes, and the evaporated water is replaced. On cooling, crystals should be formed.

6. 0.04 N sulfuric acid in carbon dioxide—free water. Cleaning of the units: After use the units are washed in a hot water current with soap and a hand brush; they are then stored in a weak acid containing some indicator. Before use they are washed several times with distilled water and are left to dry in the air.

#### PROCEDURE

The required number of units and two more for the blank analyses are dried. The glass lids are smeared with fixative. A sufficient amount of the boric acid is boiled for one minute to drive out the carbonic acid, and one drop of the indicator solution is added per cubic centimeter of solution. One cubic centimeter of the warm acid is put into the inner chamber of each

\*The original pyrex glass Conway unit is available from A. Gallenkamp, Finsbury Square, London. Borsook<sup>7</sup> recommends locally made units of glazed porcelain.

†One per cent boric acid is used instead of the 2 per cent proposed by Abelin.<sup>3</sup> The color change of the indicator in 2 per cent boric acid is not very sensitive, as in this concentration stronger polyboric acids are present (Kolthoff<sup>9</sup>).

unit, and the lid is closed. The color of the solution must be the same in all units and very near to the changing point of the indicator. The lid is displaced horizontally and 0.2 c.c. of blood or plasma is delivered from an Ostwald pipette into the outer chamber, then the lid is closed again. One-half cubic centimeter of the urease solution is now added in the same way into the outer chamber, the cover lid closed and the unit gently rotated several times to mix the blood and the urease solution. The action of the urease is completed in one minute. When the urease is added to the fourth unit, the action of the urease is already completed in the first unit, so that it is not necessary to spend time waiting for the fermentation.

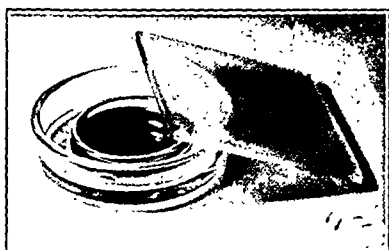


Fig. 1.

The unit is now slightly tilted to collect the fluid at one side of the chamber. The cover is now displaced so far as to allow the introduction of 1 c.c. of the potassium carbonate into the opposite side of the outer chamber. The carbonate is introduced with a pipette with a rather wide opening, an error of 10 per cent in the fluid volume being without any effect. An automatic pipette is most practicable in delivering the carbonate. After quickly replacing the lid, the unit is gently rotated ten times and then placed in an incubator at 38° C. for one hour or left at room temperature for one hour and a half. Care must be taken that the lid does not move during the placing into the incubator. The blank analyses are treated in the same manner.

Five cubic centimeters of ten times diluted urine are shaken for five minutes with 1 gm. permutit. Two-tenths cubic centimeter of the supernatant urine dilution are taken for analysis and treated exactly in the same way as the blood.

The color of the indicator changes to green during the period of the diffusion. The titration is carried out most conveniently with the Conway micro-burette.<sup>8</sup> The tip of the burette is emerged during the titration. The titration is continued until the red color does not grow any darker. With a little practice this color end point is found easily and the titration values of double determinations check within 0 to 1 mm. of the burette graduation.

#### CALCULATION

A is the number of millimeters of 0.04 normal sulfuric acid required to titrate the unknown,

B is the millimeters required to titrate the blank.

1mm<sup>3</sup>. 0.04 N H<sub>2</sub>SO<sub>4</sub> = 0.28 mg. per cent urea nitrogen in blood

1mm<sup>3</sup>. 0.04 N H<sub>2</sub>SO<sub>4</sub> = 2.80 mg. per cent urea nitrogen in urine

(1) If it is known that 1 mm. of the burette corresponds to  $x$  mm<sup>3</sup>. of sulfuric acid, the following calculation is made:

$$(A - B) \times x \times 0.28 = \text{mg. per cent urea nitrogen in blood}$$

$$(A - B) \times x \times 2.80 = \text{mg. per cent urea nitrogen in urine}$$

(2) If the volume of 1 mm. of the burette is not known, 500 mm. of the sulfuric acid are measured and titrated iodometrically with  $y$  c.c. 0.005 normal thiosulfate solution.

$$(A - B) \times y \times 0.07 = \text{mg. per cent urea nitrogen in blood}$$

$$(A - B) \times y \times 0.7 = \text{mg. per cent urea nitrogen in urine}$$

$$(3) \text{ The U:P ratio is } \frac{10 (A - B) \text{ urine}}{(A - B) \text{ blood}}$$

#### SUMMARY

The advantages of the determination of urea by the Conway unit are summarized and the technique is briefly described. It is a micromethod with very great accuracy, and a large number of analyses can be done simultaneously, the average time for one estimation being only four to six minutes.

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#### A PRACTICAL THERMOELECTRIC THERMOMETER FOR CLINICAL MEASUREMENT OF SKIN SURFACE TEMPERATURES\*

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UNTIL recently the clinical measurement of skin surface temperature has been relegated to the group of tests utilized comparatively rarely. Whether this was due to inconvenient or inaccurate methods, or to a seeming lack of importance, cannot be said, but it is becoming increasingly evident that determination of skin temperatures often facilitates diagnosis and charting the progress of certain diseases.

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Physical measurements, such as these, are important in the treatment of thromboangiitis obliterans, vasomotor neuroses, such as Raynaud's disease (Brown<sup>1, 2</sup>), arteriovenous fistula, peripheral embolism, and inflammations of the extremities. The beneficial effects of elevation and rigid immobilization in plaster casts of inflamed extremities, the skin temperatures being one of the indices of healing, have been summarized by Wangensteen.<sup>7, 8</sup>

#### SKIN TEMPERATURE MEASUREMENT

Seebeck in 1821 noticed that if a circuit is formed composed of two wires of different metals joined together at their ends, and if the junctions were at different temperatures, a thermoelectric current, proportional (to a certain point) to the difference in temperatures of the junctions, would flow in the circuit and could be measured by an interposed galvanometer. Kunkel<sup>4</sup> apparently was the first to use a thermocouple for measurement of human skin temperature.

Since the bulb expansion type of thermometer has been largely discarded for skin surface temperature measurement, the electrical resistance thermometer (Soderstrom<sup>6</sup>) and the radiometer (Hardy<sup>3</sup>) have been subjected to excellent refinements, but the thermocouple (Sheard and Williams<sup>5</sup>) retains the advantage of convenience, especially when used with one of the bronze wire double suspension galvanometers having sufficient sensitivity for use with thermocouple currents but being rugged enough to be carried about without locking the rotor.

Generally, contact methods of measuring skin temperature are inherently inaccurate because: (1) heat conduction between the thermometer and contiguous skin area will alter the reading unless the thermometer tip and skin are at the same temperature at the moment of contact; (2) the environmental air temperature affects the temperature of the uncovered contact tip; and (3) covering of the contact tip by a shielding or insulating material alters the heat equilibrium of the covered skin, changing the actual temperature at that point.

The above sources of error are held to a minimum by a properly designed skin thermometer having sufficient accuracy for clinical observation, if not for exact quantitative measurements, by (1) keeping the mass of the contact tip very low so conduction error is negligible; (2) using an anaerobic tip in contact with the skin; and (3) employing a thermometer designed with a very low lag; namely, one which gives an almost instantaneous reading, permitting movement of the tip along the skin, maintaining its contact.

In the routine measurement of skin surface temperature for clinical purposes in the University Hospital, we have used a portable thermocouple and galvanometer, accurately calibrated, which comes nearest to fulfilling the above requirements, the galvanometer reacting almost instantaneously to temperature changes in the tip. The apparatus, which is much more sensitive than clinical use necessitates, is compactly fitted into a box which is conveniently carried about by hand (Fig. 1).

The simple thermocouple junction applicator diagrammed in Fig. 2 has adequately met the technical conditions outlined and has proved highly satisfactory in diagnostic work. It consists of a piece of pernambuco or balsa

(any highly cellular substance would be adequate), measuring 8 by 1.5 by 1.5 cm. One end is beveled to permit its being applied at an angle, and a hole 4 cm. in depth of sufficient diameter just to accommodate the rubber sheath for the thermocouple wires is bored from the rectangular surface to the beveled end.



Fig. 1.—Portable thermoelectric skin temperature cabinet, showing manner of applying warm junction.

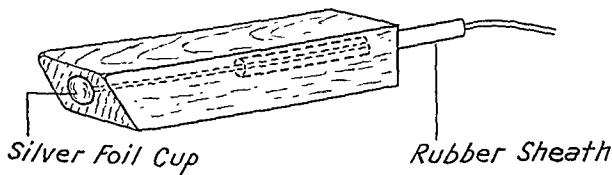


Fig. 2.—Diagram of warm junction applicator.

The previously soldered tips of the thermocouple wires (2 36G copper and 4 36G constantan) are threaded on a Keith abdominal needle and carried through the tunnel and remaining 4 cm. of wood to the beveled end. The concave surface of a pounded silver foil cup, 4 mm. in diameter and 1 mm. deep, is soldered lightly to the wire junction, and the wires are drawn taut to bring the cup snugly against the end of the applicator. By making the tip of such negligible mass, temperature equilibrium is attained almost instantly, the convex surface of the tip permitting its rapid movement along the skin from point to point.

The rate of equilibrium with room air at  $27^{\circ}\text{C}$ . of the warm junction after being exposed to a skin temperature of  $34^{\circ}\text{C}$ . is plotted in Fig. 3, galvanometer readings being taken every ten seconds. It will be seen that an exponential form of curve is obtained, a drop of over 3 degrees being obtained in the first ten seconds, thermal equilibrium having obtained in sixty seconds. For ordinary skin temperature measurements, where adjacent areas vary within 2 or 3 degrees, readings may be taken within five seconds after the junction is applied. Elevation of the warm junction temperature from a room temperature of  $27^{\circ}\text{C}$ . to a skin surface temperature of  $34^{\circ}\text{C}$ . takes place within ten seconds.

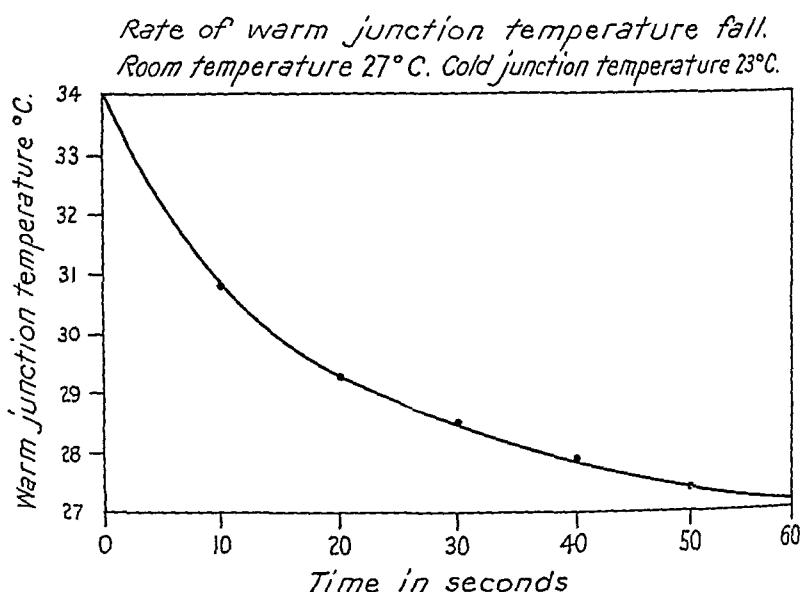


Fig. 3.—Rate of warm junction temperature fall after application to skin at  $34^{\circ}\text{C}$ . Room temperature  $27^{\circ}$ .

#### SUMMARY AND CONCLUSIONS

The importance of skin surface temperature measurements in certain surgical conditions has been pointed out. Data so obtained are helpful in diagnosis and evaluation of treatment in thromboangiitis obliterans, neurovascular diseases, arteriovenous fistula, embolism, and inflammations.

A new form of warm junction applicator for use with a handy, portable thermoelectric thermometer is described for routine clinical determinations of skin surface temperatures, the device being offered to meet the technical objections attending the use of contact methods.

Grateful appreciation is extended to Dr. Irwin Vigness, of the Department of Radiology, whose interest and cooperation was largely responsible for the construction of the apparatus.

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## PHOTOELECTRIC PLETHYSMOGRAPHY OF ANIMAL TISSUES\*

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SEVERAL photoelectric plethysmographs have been described for the study of vascular reactions in the animal, notably in the rabbit's ear.<sup>1,2</sup> The application of the idea of photoelectric plethysmography to the study of the peripheral circulation in man<sup>3-5</sup> met with such success that it seemed worth while to explore further its possibilities in the animal. The technique described below was suggested by the method developed for the nasal septum.<sup>3</sup> It has the advantages of extremely high sensitivity, freedom from inertia and consequent distortion, ease of application of the plethysmograph, and relief from the irritation and errors associated with the mechanical type of plethysmograph.

The validity of the photoelectric plethysmogram and the sources of error involved have been discussed in previous papers.<sup>4,5</sup>

Fig. 1 shows an arrangement of the photoelectric plethysmograph having a wide application in animal experiments. The tissue, whose vascular reactions are to be studied, is transilluminated by a pencil-type flashlight bulb. It is best to operate the bulb slightly below its rated voltage on an accumulator, to avoid a slowly shifting illumination intensity due to bulb deterioration or falling battery voltage. Either of these shows up in the plethysmogram as a gradual increase in the apparent opacity of the tissue. Light which has escaped absorption in the tissue is reflected into the photocell housing by the mirror placed at the end of the tube. Although the photocell may be placed directly beneath the tissue in some instances, the use of a mirror adds greatly to the convenience and flexibility of the technique. By using interchangeable tubes varying in diameter, the mechanical requirements of different situations may be conveniently served.

Semiquantitation of the plethysmogram is provided by comparing the changes in the plethysmogram with the deflection resulting from absorbing

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a fraction of the light with a filter. This is simply a sheet of thin glass (cover slip for microscope slides) on the filter carrier. During the recording of the plethysmogram, the filter carrier is quickly flipped into place, thus inserting the filter in the light path. The validity of this procedure has been discussed previously.<sup>4, 5</sup> Quantitation is also provided by the grid bias control described below.

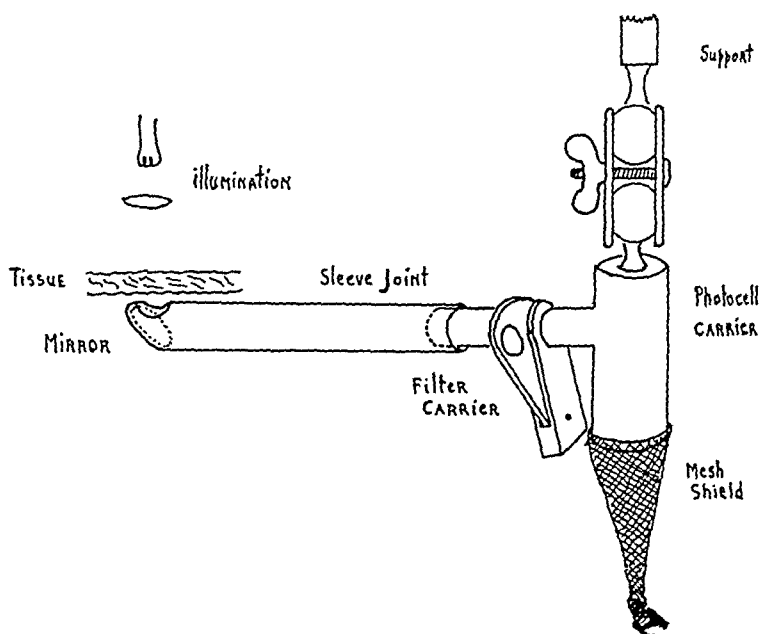


Fig. 1.—Photoelectric plethysmograph for animal tissues.

*Technical Data on Amplification.*—Although the use of amplification complicates photoelectric plethysmography, there are good reasons for employing it. Amplification eliminates the need for intense illumination with its possible heating effects and makes possible the use of high frequency, low sensitivity recording instruments. A suitable amplifier must be free from drift, and provide linearity of response as well as a wide range of amplification. The amplifier meeting these requirements most satisfactorily is a modification by Dr. H. B. Peugeot, of the standard two-stage direct coupled type (Fig. 2), employing 6SJ7 and 1852 tubes and delivering 0.35 Ma. per millivolt at full sensitivity, a gain which allows adequate variations in recording amplification. When these high Mu tubes are operated at less than their rated maximum plate and screen grid voltages, drift is greatly reduced. Further reduction of drift is obtained by eliminating thermocouple effects on the bias of the first tube by building it in a separate cabinet, connected to the amplifier proper by means of a Banana Jack. Further stability is secured after the tubes have been in use for several days due to the smoothing of the cathode with use, giving more constant emission of electrons. The cabinets are galvanized iron; all leads are shielded cable. Both are grounded. By allowing the amplifier to heat for at least an hour to reach thermal equilibrium, drift is less than 4 mm. per hour on the record when the sensitivity is sufficient to give a volume

pulse wave of 1 cm. As has been indicated above, drift on the record may be due to deterioration in illumination which shows up as apparently increasing opacity.

The amplifier is set by balancing the second stage by means of its grid bias ( $GB_2$ ) with the volume control switch ( $VC$ ) off until the plate current reads zero. The volume control switch is then turned on, and the balancing is repeated by means of the grid bias of the first stage ( $GB_1$ ), the volume control being set at any desired amplification. The amplification will follow accurately the curve of the volume control potentiometer and calibration is therefore possible. However, it is usually necessary to readjust the bias of the first stage when changing amplification, particularly if the change is large.

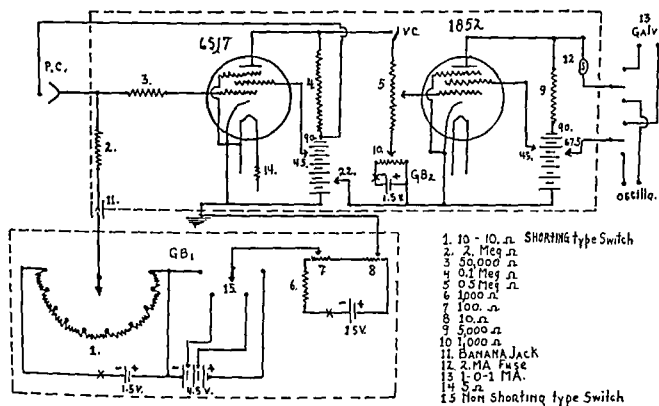


Fig. 2.—Two-stage direct-coupled amplifier for photoelectric plethysmography. P.C. Photoelectric cell, cathode to grid.  $GB_1$ ,  $GB_2$ . Grid bias controls for first and second stages. Oscillo-oscillograph or string galvanometer.

The grid bias control on the first stage serves as an indicator of the input from the photocell and, therefore, of the changes in the intensity of the light reaching the photocell. This fact may be used instead of the filter to calibrate the plethysmogram in arbitrary units. Thus, by adjusting the volume control until a unit change in the input produces a unit deflection in the plethysmogram, several simultaneously recorded plethysmograms may be compared with respect to the size of the vascular changes.

This amplifier may be used with recording galvanometers, such as the General Electric type A, G6 or G7, Westinghouse 877-121, or the Hindle string galvanometer. The latter is not as satisfactory as galvanometers with fixed sensitivities, since the string tension and, therefore, the sensitivity, may change without notice. Further, the string galvanometer requires shielding or it serves as a feed-back of alternating current into the system. It is important to note that the shunt resistance 9 on the plate circuit of the second stage should have approximately the same value as the recording instrument.

Photoemissive cells, similar to the G. M. Laboratories 51A, are suitable. The small cells are to be preferred because of ease in manipulation. It is very important to shield the photocell. This is done by means of a galvanized iron mesh cone which fits well up on the photocell carrier which is also made of galvanized iron. Induction difficulties are thus avoided.

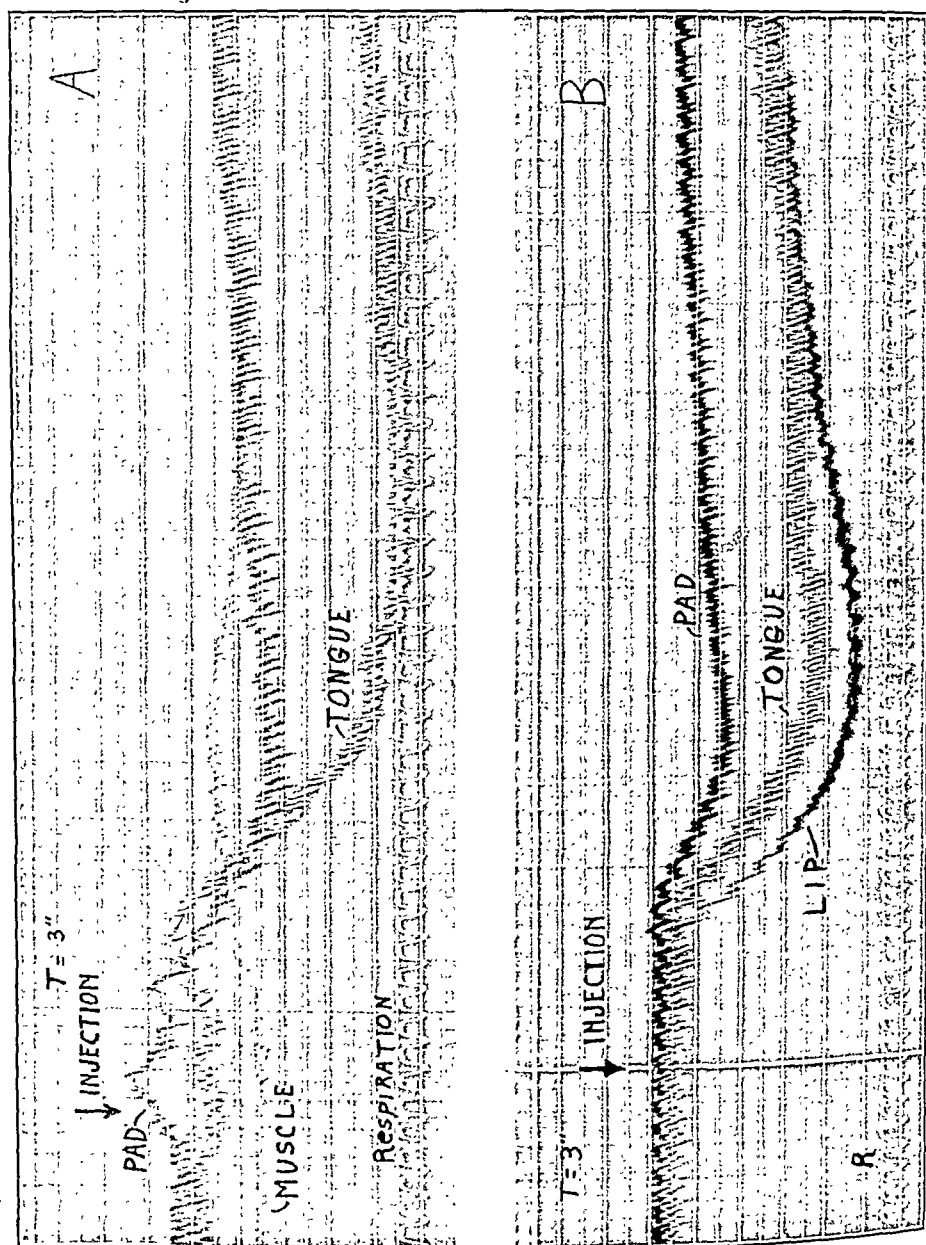


FIG. 3.—Plethysmograms of the foot pad, thigh muscle, tongue and foot pad of a dog. Intravenous injection of 0.5 mg. adrenalin per kilogram body weight.

Illustrative plethysmograms are provided in Fig. 3, showing the effects of intravenous injections of adrenalin on the vascular areas in the tongue, lip, thigh muscle, and foot pad of the dog. Vascular changes in the latter area were recorded with the skin plethysmograph<sup>5</sup>, those in the tongue, lip, and thigh muscle with the technique previously described. Well-known constrictor

and dilator actions of adrenalin are seen in these responses. It is interesting to note the strong constrictor action in the tongue despite the presence of striated muscle. This may be related to dominant effects on the mucosa and on arteriovenous anastomoses which have been found in the dog's tongue.

#### SUMMARY

A description of the application of photoelectric plethysmography to animal tissues is given. A suitable amplifier is designed which meets the requirements of freedom from drift, linearity, and wide range of amplification. The technique has adequate sensitivity for the study of vascular reactions in such limited areas as the tongue, lip, eyelid, skeletal muscle, etc. It is likewise applicable to arteriography.

We are greatly indebted to Dr. H. B. Peugnet, of the Washington University School of Medicine, for important aid in designing the amplifier described in the paper.

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#### A METHOD OF EMPLOYING HORSE PLASMA AND HEMOGLOBIN AS ENRICHMENTS IN PRIMARY GONOCOCCUS ISOLATIONS\*

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EVER since Neisser first observed the gonococcus in 1879, many investigators have taken up the problem of a suitable medium on which the gonococcus would grow consistently on first culture from the human body. It was not until 1885 that Bumm<sup>1</sup> succeeded in growing and isolating the organism on coagulated human blood serum. Wertheim,<sup>2</sup> deciding that the growth of the gonococcus was tied up with human blood albumin, first produced a serum agar. He emulsified his culture material in human serum, diluted this emulsion with more serum, then mixing this with melted agar cooled to 40° C., he poured plates. Kiefer<sup>3</sup> brought the culture method closer to present-day procedures by first pouring the serum plates and then inoculating them with the culture material.

It was soon discovered, however, that serum did not give dependable results, and other sources of albumin were sought. It was then that ascitic and hydrocele fluids were tried. These were also found to be of uncertain

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value. Ascitic fluid, to be of service, must be bile free, of high specific gravity, and sterile. When contaminated, which it often is, it is difficult to sterilize, since filtering it removes most of its growth-inducing substances and heat coagulates the albumins. It also has a tendency to grow more alkaline and to deteriorate on standing. Abel,<sup>4</sup> therefore, went back to the use of blood, employing Pfeiffer influenza plates for gonococcus isolations. At first, human blood only was used, then rabbit blood, and finally Koch<sup>5</sup> showed that horse blood also was suitable.

Many and various media were then elaborated and tried, and the advantages of serum and red blood cells, as enrichments, were studied. It was found that red blood cells stimulated initial growth of gonococcus and that the presence of serum produced more luxuriant growths (Cole and Lloyd<sup>6</sup>). Animal tissues, such as beef heart and steak, were then substituted. But since in the preparation of the medium a large part of the necessary growth elements were lost because of heating and filtration, these media required other enrichments, such as egg (Huntoon<sup>7</sup>) or blood.

About 1920, the Levinthal medium, originally used for the influenza bacillus, came to be considered by many the most reliable medium for initial gonococcus isolations. This clear and colorless cooked blood agar was employed by Galli-Valerio,<sup>8</sup> by Cohn, by Strempel,<sup>9</sup> and others. Ruys<sup>10</sup> used it successfully with the addition of ascitic fluid. Later Cohn<sup>11</sup> modified it further, using veal infusion agar, ascitic fluid, and a filtered, veal broth cooked blood extract.

Media enriched with blood seem to have given to many investigators the most consistent results. Jenkins<sup>12</sup> found that citrated plasma agar alone gave satisfactory cultures. After testing many media and elaborating a special agar, Tulloch<sup>13</sup> obtained the best results with initial cultures when he added human or rabbit plasma plus one drop of whole human blood for each plate. Later Marshall<sup>14</sup> used a medium consisting of nutrient agar plus 5 to 10 per cent precipitated human red blood cells. Also Casper,<sup>15</sup> employing Bieling's method for the preparation of a pneumococcus medium, applied it to the gonococcus. He enriched an agar with horse blood, which was first hemolyzed by allowing a volume of blood to flow directly from the animal into two volumes of sterile distilled water. More recently some investigators obtained satisfactory initial gonococcus growths by the use of various chocolate agars (McLeod and co-workers,<sup>16</sup> 1934; Carpenter and co-workers, 1936<sup>17</sup> and 1938;<sup>18</sup> Reitzel and Kohl,<sup>19</sup> 1938).

From this brief survey of the literature, the important role which blood has played in the development of various gonococcus media becomes apparent. Media in which blood was used as enrichment seem to have been the simplest to prepare and the most reliable to use, since even dehydrated prepared agar, when enriched with it, gave good initial growth of the gonococcus (Reitzel and Kohl<sup>19</sup>).

However, the ideal medium for gonococcus isolations should be not only dependable and easy to prepare, but also one on which the characteristics of the gonococcus colony can be easily distinguished. It should, therefore, be colorless and transparent. In rendering blood media transparent and colorless, heating and filtering not only complicate the preparation of the medium,

but also greatly reduce the essential growth elements. As shown by the experiments of Torrey and Buckell,<sup>20</sup> the growth-stimulating substances of red blood cells are readily absorbed by materials ordinarily used to clarify media. On the other hand, Thjötta and Avery<sup>21</sup> found that even moderate heating impaired the growth accessory "V" factor essential for the cultivation of hemophilic bacilli. Wright<sup>22</sup> has also shown that overheating body fluids renders them inhibitory to the growth of the pneumococcus and other organisms.

It has been possible to prepare, in our laboratory, a simple and efficient medium consisting of veal infusion agar enriched with uncooked and unfiltered constituents of sterile citrated horse blood, namely, plasma and hemoglobin. The proportions for this plasma hemoglobin agar are 25 c.c. of veal infusion agar, 3 c.c. of sterile plasma, and 1 c.c. of clear hemoglobin in distilled water.

In preparing the veal infusion agar, the method described by Park and Williams<sup>23</sup> is followed.

The horse plasma is obtained by decanting with sterile precautions from whole citrated horse blood after the red blood cells have completely settled. Free hemoglobin may be obtained by bleeding the animal directly into an equal volume of sterile distilled water, or by mixing whole horse blood with an equal amount of water and keeping in the water bath at 45° C., with occasional shaking, until hemolysis takes place. The remaining red blood cells, from which the plasma has been decanted, may be diluted with 5 volumes of sterile distilled water which often causes hemolysis of the cells. If hemolysis does not then occur, the addition of a few drops of ether will lyse the red blood cells. There is good reason to believe that rabbit blood can be used almost as well as horse blood.

This plasma hemoglobin agar plate is transparent, practically colorless, since the reddish tinge fades in the incubator overnight, and it may be prepared the day before it is to be used. However, on freshly poured plates, the colonies are somewhat larger. We have, on at least two occasions, used plates which were five days old, and they have good growth of gonococci, though the colonies were small. Forty-eight hours after inoculation and incubation at 37° C., in an atmosphere containing ten to twelve volumes of carbon dioxide, the gonococcus colonies have the typical ground glass appearance and seem transparent when the plate is held up and light passes through them. Macroscopically the colonies usually appear glistening, round, raised, and with entire edge. Older colonies are usually irregular in shape and edge. There is, as a rule, a good growth of gonococci at the end of twenty-four hours, when grown under the above conditions. The viability of the gonococcus on this plate has been tested only up to one week. The transplants from week-old cultures all grew well.

The question whether cultures of all positive cases grow on a medium is a vital one. The only check the laboratory has on the efficacy of a medium is its comparison with the smear diagnosis and other known good media. The method of obtaining cultures from the patient, the transportation of the cultures to the laboratory, and the amount of inoculum planted on the plates are important considerations when evaluating primary gonococcus cultures on a medium.

Table I shows results obtained on freshly poured plasma hemoglobin plates compared with other good media and with smears.

TABLE I

NO. POSITIVE CASES	NO. POSITIVE ON PLASMA HEMOGLOBIN AGAR	NO. POSITIVE ON DIFCO AGAR*	NO. POSITIVE ON HORSE BLOOD WATER AGAR†	GRAM-STAINED SMEARS		
				POSITIVE	SUSPICIOUS	NEGATIVE
Vulvovaginitis	38	33	Not done	28	7	3
Vulvovaginitis	120	Not done	113	89	18	13
Male	44	42	44	42	0	2

\*Difco proteose agar No. 3 and Difco dehydrated hemoglobin.

†Three parts of veal infusion agar plus 2 parts of horse blood water (equal volumes of horse blood and sterile distilled water).

Identifications on 2 per cent dextrose, 2 per cent maltose, and 2 per cent levulose agar plates, also on plain agar and 5 per cent washed blood agar, were carried out on all the strains included in Table I. Since the gonococcus colonies are more easily distinguished from similar colonies when grown on transparent and colorless media, fishings were usually made from the plasma hemoglobin plate before the oxydase test<sup>24</sup> was performed. This proved to be of importance because, when the dye was poured on a culture plate containing very few gonococcus colonies and a large growth of other flora, organisms were washed over the plate making pure cultures for identification impossible and often causing their loss. However, the oxydase test is clearly defined on the medium. The color changes are especially well seen when the plate being tested is placed on a white sheet of paper or held up to the light.

#### SUMMARY

An agar medium for primary gonococcus cultures is prepared by the use of veal infusion agar, horse plasma, and hemoglobin.

The advantages of the medium are:

1. It is efficient and easily prepared.
2. It is transparent and almost colorless.
3. The characteristics of the gonococcus colony on this medium are more easily distinguished from similar colonies.

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## ISOLATION OF BACTERIA BY SURFACE-STROKE PLATING\*

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IN THE course of some twenty-five years of teaching, the technique most essential to successful bacteriologic work, namely, the easy and practical isolation of pure cultures, is the very point least often stressed, or illustrated, in textbooks and laboratory manuals. This procedure is commonly passed over with a few words on "sun-burst" or parallel stroking of cultures on agar plates.

Furthermore, we have observed that the average student has more difficulty with successful colony isolation than with any other common bacteriologic routine. A method has been evolved in the attempt to overcome this deficiency. Its use in our hands, both personally and in classes, has yielded such constant and almost unvarying success that we feel that its use may well be advocated for trial by others who may have had similar difficulty. The procedure is illustrated in Fig. 1.

(1) In this method sterile agar plates are poured and well solidified. Using, preferably, a smooth platinum loop sealed in a light, hollow glass handle, a loopful of mixed culture (as from a Loeffler's throat culture) is carried to section A, and a small spot or smear is made at point marked "begin."

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(2) The loop is flamed to remove excess material, cooled, then stroked in parallel over approximately one-third of the plate area. The initial smear is touched only in the first three or four strokes.

(3) The plate is then rotated counterclockwise one-quarter turn, and the same area (A) is cross-stroked—again avoiding the initial spot of inoculation.

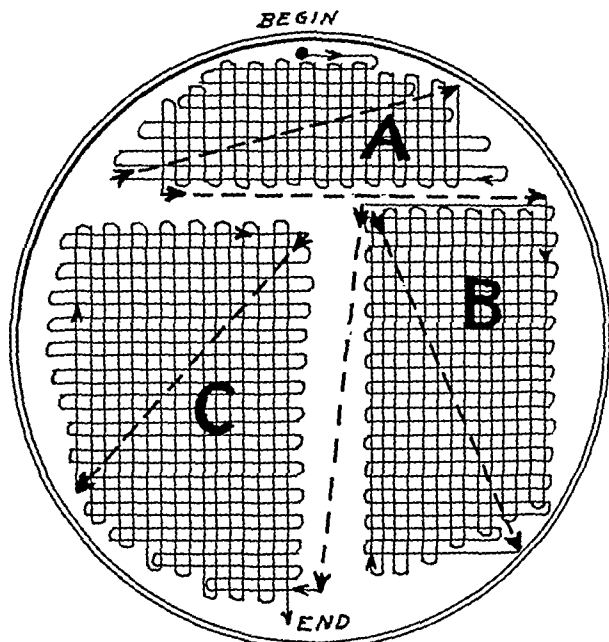


Fig. 1.

(4) Having cross-stroked section A, the loop is carried up to section B, and stroking is repeated—now avoiding contact with strokes in section A.

(5) Again the plate is rotated one-quarter turn, and section B is cross-stroked.

(6) The loop is then carried to section C and stroked—now avoiding contact with section B.

(7) The plate is turned the final one-quarter turn, and section C is cross-stroked.

The entire operation is accomplished in only some thirty seconds' time. Depending upon the numbers of bacteria placed on the original spot of inoculation, the plates invariably show optimum colony distribution in some section of the plates.

In 1934 we described a rotary inoculating table<sup>1</sup> which is still considered most useful for colony isolation. However, in the absence of this device, we feel that the method herein described will be found to be more uniformly successful than any of the procedures commonly used, either in private laboratory work, or in the instruction of beginning students.

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## ANAEROBIC CULTIVATION AS A ROUTINE BACTERIOLOGIC PROCEDURE IN THE CLINICAL LABORATORY\*

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IT WAS until recently a frequent experience in our clinical laboratory to observe microorganisms in smears of the original material which subsequently failed to develop in culture. Occasionally, it was necessary for the laboratory to submit an essentially negative report. In an effort to overcome the inadequacy of our cultural methods, a systematic attempt has been made to eliminate the incidence of negative cultures from specimens known to have contained bacteria.

There are at least four acceptable explanations for the failure of microorganisms to develop in primary culture. It is possible, for instance, that the specimen, when received, contained only dead bacteria, or that the amount of inoculum is inadequate. Nevertheless, it would seem more likely that the media are lacking in certain essential nutritive ingredients, or that the oxygen tension during incubation is too high. Preliminary trials with various culture media indicated that the nutritional factor played an important role but, at the same time, not the fundamental one. As a result, the comparative value of strictly anaerobic cultivation in the primary isolation of bacteria from clinical sources has been investigated.

Within the last few years many authors (Colebrook, 1930; Harris and Brown, 1929; McDonald and others, 1937; Schwarz and Dieckmann, 1927; Sears and Vinton, 1936) have called attention to the importance of anaerobic nonhemolytic streptococci. Topley and Wilson (1936) pointed out that anaerobic cultivation of primary agar plate cultures yields a higher incidence of hemolytic streptococci than do the corresponding aerobic plates. Smith and associates (1938) report a case of meningitis caused by a strictly anaerobic hemolytic streptococcus, and conclude that anaerobic cultivation of routine hospital material is important. Smith (1936) reported studies upon obligate anaerobic pneumococci. An interesting account of the sensitivity to oxygen of *Past. pestis* and other members of the *Pasteurella* genus was that of Schütze and Hassanein (1929). Furthermore, Dixon and Deuterman (1937) recently called attention to the possible pathogenic role of the members of the anaerobic genus, *Bacteroides*. It is evident, then, that, in addition to the anaerobes of war-wound infections, the importance of anaerobic, or reduced oxygen, cultivation in primary culture is amply indicated by the pertinent literature. Nevertheless, there appears to be little information regarding the prevalence of anaerobes in unselected routine cultures.

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The present report deals with the results obtained by comparing duplicate aerobic and anaerobic primary cultures on blood agar plates. A semi-solid medium was also included in a small series. A total of approximately 2,500 consecutive routine specimens were examined by the method to be described. Of these 1,847 produced positive cultures.

*Experimental Procedure.*—Each specimen, as received, was streaked in an identical manner upon the surface of two blood agar plates. One Petri plate was labeled aerobic; the other was designated as the anaerobic culture and placed in a jar for anaerobic incubation. Some of the anaerobic plates were immediately subjected to anaerobiosis, but more often an interval of one to two hours intervened. The following morning the aerobic and anaerobic plates were examined together, and the resulting growths were compared. With a few exceptions all cultures were incubated for a minimum period of five days at 37° C., during which time they were examined daily for the appearance of new colonies. Further incubation depended upon the diagnosis submitted and the nature of the specimen.

No attempt was made to use selected specimens. Wherever specific culture methods were indicated, as in the isolation of *N. gonorrhoeae*, *Br. abortus*, or *C. diphtheriae*, the usual blood agar plates were inoculated in addition. All throat and feces cultures were included in the recorded series, but repeat specimens from the same patient were omitted. Since the use of agar plates has not been routine in the preparation of blood cultures, this type of specimen has not been considered. As a group, then, the specimens, upon which the present report is based, are representative of those received by any large hospital laboratory with the possible exception of bronchial secretions, which constituted 10 per cent of the cultures in our series.

*Method of Producing Anaerobiosis.*—To be practicable for the clinical laboratory an anaerobic procedure must be rapid, efficient, safe, and capable of accommodating a large number of cultures at one time. Furthermore, it is essential that such a method permit the use of agar plates, since only by the development of surface growth can a mixture of microorganisms be quickly and easily identified.

None of the many anaerobic methods known to us seems ideal. Toward that end one of us (E. H. S.) is devoting considerable effort, which will be made the subject of a future publication. In 1937 Weiss and Spaulding suggested a technique involving partial evacuation of a jar and the subsequent refilling with hydrogen catalyzed by palladium. Certain modifications in the technique and apparatus, as described below, have, we believe, resulted in a more practicable procedure. The data herein reported were obtained by this technique.

The essential system of two T tubes, a two-way stopcock (A) and a three-way stopcock (B) with downward outlet at end of the stopper, is illustrated in Fig. 1. A three-way stopcock, similar to A. H. Thomas Co., No. 9294, with 4 mm. bore is satisfactory. The glass apparatus may be suspended by metal clamps on ring stands or, preferably, placed in a grooved board and held in

place by metal strips. In place of an open mercury manometer, as originally described, a vacuum gauge has been substituted. Two types of gauges, especially designed for use with this apparatus, are available.\* The first is subject to changes in atmospheric pressure, but is relatively inexpensive; the second is contained in a pressure-tight case and may be relied upon regardless of altitude or pressure. Both gauges have extra markings at 76, 640, and 710 mm. negative pressure. The gauge is attached to the glass system, as indicated in Fig. 1.

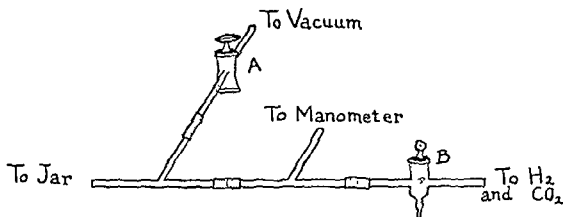


Fig. 1.—Drawing of the basic system of the anaerobic apparatus

A Cenco-Hyvac, or similar oil pump, provides a rapid means of evacuation and is highly desirable where a large number of cultures must be handled. For the smaller laboratory, however, the slower but inexpensive water suction pump (at least  $5\frac{3}{8}$  inch length) will suffice. The pump is attached to the system by rubber pressure tubing, as indicated in the diagram.

Any jar of convenient shape and size may be used providing it can withstand the degree of vacuum required and is equipped with at least one stopcock. Several types of anaerobic jars are available, but they are usually expensive. The Hempel desiccator has proved satisfactory, but is not designed for convenient storage in the average incubator. We employ at present a chamber consisting of a glass cylinder (A. H. Thomas Co, No. 1085-A, flange at least  $\frac{1}{2}$  inch diameter) and a flat aluminum alloy cover with one stopcock extending horizontally from the side of the top. In a jar of this design the maximum of space is utilized. It will hold 14, 100 by 10 mm. plates and 25, 120 by 16 mm. culture tubes at one time. The inexpensive metal top is being manufactured at present by the Temple University machine shop.

The reduction of oxygen is accomplished by the catalytic power of palladium impregnated upon asbestos shreds. In this highly active form heat is not required, thereby minimizing the danger of explosion. It may be purchased from supply houses as 5.0 per cent palladiumized asbestos.

The procedure is carried out in the following manner: The inverted agar plates are placed in the jar, together with a flat porcelain dish (69 mm. diameter) containing 2 gm. of palladium. The catalyst should be located near the gas inlet. The criterion of satisfactory anaerobiosis is the reduction of methylene blue. The dye is added in a final concentration of 1:60,000 to meat extract broth, pH 7.6, containing 2.0 per cent dextrose. A small piece of thymol is

\*United States Gauge Co., 522 Penfield Building, Philadelphia.

added as a preservative. It has been found convenient to keep the cotton-stoppered tube attached to the side of the jar with adhesive tape. The ground surface of the cover and of the cylinder are well smeared with a grease, such as Lubriscal (A. H. Thomas Co.) or a mixture of Cello-Seal and Cello-Grease (Fisher). The cover is put in place, and with pressure is rotated about the flange of the cylinder to produce a satisfactory seal. The jar is attached to the system by means of a rubber connection. With stopcock B closed, as in Fig. 1, and stopcock A open to the system, evacuation begins. This is continued until the manometer registers 710 mm. Turn stopcock A to disconnect the source of vacuum and to connect the system beyond stopcock B with carbon dioxide tank by means of rubber tubing. Without turning stopcock B rinse out the hose with the gas, shut off carbon dioxide tank, and open stopcock B by a 90 degree turn. The jar is now connected with the gas tank. Admit carbon dioxide until the gauge records a reading of almost zero (fifteen to thirty seconds). Close stopcock B and open A. When evacuation has again reached 710 mm., turn stopcock A and observe vacuum gauge for at least fifteen seconds for signs of a leak. If the pressure remains stationary, connect rubber hose to the hydrogen tank. It is more convenient to maintain a permanent connection with both gas tanks by inserting a Y tube in the rubber hose between stopcock B and the source of gas. Rinse out the tube through the outlet in stopcock B as before. Open stopcock B and admit the gas slowly; between thirty seconds and one minute should be consumed in refilling the jar. Turn the stopcock on the jar and disconnect from the system. The jar is now ready for the incubator. It may be desirable to use a clamp to hold the top in place. If, however, care is taken to keep the jar in a vertical position, this is not necessary.

The following morning the indicator should be colorless. To remove cultures, open stopcock, slide the cover off the cylinder, and remove the catalyst immediately. The top should never be put back on the jar unless the catalyst has been removed. An explosion may result. The catalyst should be heated over a Bunsen burner for five minutes to drive off moisture and then, upon cooling, is again ready for use.

By use of partial evacuation (710 mm. Hg) inversion of agar plates is possible. Because the technique is safe, rapid, and apparently efficient, its application to routine procedure appears justified. The apparatus above described has also been found ideal in producing an atmosphere of 10 per cent carbon dioxide as used in the isolation of *Brucella*, gonococci, and other carbon dioxophilic bacteria.

*Media Employed.*—A total of 1,358 positive cultures were obtained upon a beef infusion blood agar prepared according to Wright (1933). The value of this medium, especially for the isolation of fastidious pathogens, is believed to be due in part to the reduction of certain oxidized constituents in the peptone. A second series of 489 positive cultures were examined after isolation upon a nutrient blood agar containing 1.0 per cent Bactopeptone, 0.3 per cent Difco meat extract, and 0.5 per cent sodium chloride to which was added 6.0 per cent human citrated blood. It was hoped that, by comparing media of

two different growth abilities, the relationship between the nutritive quality of the medium and the oxygen requirements of these bacteria might be indicated. All media were used within twenty-four hours after preparation.

*Experimental Results.*—The comparative aerobic and anaerobic growths upon both types of media are presented in Table I. No consideration is given to the various species present.

TABLE I

COMPARISON OF THE NUMBER OF POSITIVE CULTURES ON AEROBIC AND ANAEROBIC BLOOD AGAR

AEROBIC PLATE	ANAEROBIC PLATE	MEAT EXTRACT BLOOD AGAR		BEEF INFUSION BLOOD AGAR	
		NO. OF SPECIMENS	PERCENTAGE	NO. OF SPECIMENS	PERCENTAGE
+	+	373	76.3	1223	90.0
+	0	24	4.9	16	1.2
0	+	92	18.8	119	8.8
Total		489	100.0	1358	100.0

Since Wright's medium contains an infusion base and is prepared in such a manner as to contain reduced substances, one might expect, in comparison with the meat extract agar, to find a less striking difference between the aerobic and anaerobic plates. Table I supports this expectation. On the meat extract medium, 18.8 per cent of the specimens produced positive cultures only by anaerobic cultivation, whereas the corresponding figure on infusion agar was 8.8 per cent. Since the series included a large proportion of feces specimens in which negative cultures never occurred, this figure assumes added significance. Furthermore, it is our belief that the figure of 8.8 per cent represents those specimens which, prior to the inauguration of anaerobic cultivation, remained sterile in spite of positive microscopic evidence. On the other hand, a surprisingly small number of specimens produced aerobic growth exclusively.

TABLE II

ANALYSIS OF SPECIMENS SHOWING ONLY ANAEROBIC GROWTH

TYPE OF ORGANISM	NUMBER OF ISOLATIONS	
	MEAT EXTRACT BLOOD AGAR	BEEF INFUSION BLOOD AGAR
Hemolytic streptococcus	40	50
Nonhemolytic streptococcus	37	30
Pneumococcus	13	16
Gram-negative cocci	21	15
Bacteroides	7	14
Diphtheroids	8	11
H. influenzae	0	3
Miscellaneous	11	10

*Analysis of Specimens Containing Only Anaerobes.*—If the anaerobes isolated from aerobic-negative specimens consist only of contaminants and non-pathogenic types, there would be considerable doubt as to their routine value in the clinical laboratory. An examination of Table II, however, reveals that the most common organism recovered under these conditions was the hemolytic streptococcus. In order of decreasing frequency were the nonhemolytic strep-

tococci, a heterogeneous group of gram-negative cocci, pneumococci, and bacteroides. It is of interest that the *Clostridium* group was only occasionally encountered among these specimens.

*Anaerobic Hemolytic Streptococci.*—Because of the importance of hemolytic streptococci in human infections, the incidence of anaerobic hemolytic streptococci in the entire series of specimens has been tabulated. The results appear in Table III.

TABLE III  
HEMOLYTIC STREPTOCOCCI  
Frequency of Anaerobic Isolations

AEROBIC PLATE	ANAEROBIC PLATE	MEAT EXTRACT BLOOD AGAR		BEEF INFUSION BLOOD AGAR	
		NUMBER OF SPECIMENS	PERCENTAGE	NUMBER OF SPECIMENS	PERCENTAGE
+	+	71	33.2	270	56.3
+	0	5	2.3	4	0.8
0	+	138	64.5	206	42.9
Total		214	100.0	480	100.0

It will be seen by examining Table III that more than 40 per cent of the hemolytic streptococci isolated on infusion blood agar failed to appear on the aerobic plates. The anaerobic strains originated from many different pathologic conditions, and in no way suggested a predilection for any one part of the body or disease process. No attempt has been made to determine the serologic grouping of these strains.

The high percentage of anaerobic hemolytic streptococci occurring in the meat extract series may be accounted for by the use of a medium of low nutritional value. Since almost 65 per cent of the isolations were recovered only on the anaerobic plate, it would appear that anaerobiosis may, to some extent, compensate for a poor culture medium.

An observation of considerable practical importance has received no consideration in compiling these data. It has been customary to observe the development of hemolytic streptococci, and sometimes pneumococci, within twenty-four hours on the anaerobic plates, whereas the corresponding aerobic plate might require two or three days before revealing similar colonies. Since growth occurred under both conditions, it was tabulated accordingly, but the practical significance of the more rapid anaerobic growth is obvious. Not only did many of the anaerobic cultures develop earlier, but, in addition, they produced more luxuriant colonies and larger areas of hemolysis.

*Anaerobic Pneumococci.*—The isolation of pneumococci, which at the same time failed to appear on the primary aerobic plate, was not an uncommon occurrence. In many instances, but not all, these organisms were identified biochemically and typed by the Neufeld reaction. The "greening" of blood agar and hemolysis tend to be suppressed, but to a varying degree reappear upon exposure to air. An outstanding instance in which an anaerobic culture resulted in the early institution of therapy was a type VII pneumococcus meningitis. In spite of negative microscopic evidence of bacteria in the original material, the anaerobic plate after eighteen hours' incubation revealed several

colonies which were immediately typed. The aerobic plate, as well as those from succeeding specimens of spinal fluid, remained uniformly sterile. The data relating to the isolation of pneumococci on both types of media are presented in Table IV.

TABLE IV  
PNEUMOCOCCI  
Comparison of Duplicate Aerobic and Anaerobic Blood Agar Plates

AEROBIC PLATE	ANAEROBIC PLATE	MEAT EXTRACT BLOOD AGAR		BEEF INFUSION BLOOD AGAR	
		NUMBER OF SPECIMENS	PERCENTAGE	NUMBER OF SPECIMENS	PERCENTAGE
+	+	53	57.6	227	82.9
+	0	16	17.4	22	8.0
0	+	23	25.0	25	9.1
Total		92	100.0	274	100.0

*Other Anaerobic Types.*—In the infusion series were 213 isolations of various nonhemolytic streptococci. Of these 152 appeared on both plates, but 59 were anaerobic only. No attempt to classify these strains according to Prévot (1925) has been made. It is interesting also that of 28 cultures of *H. influenzae* recovered, 13 appeared only with reduced oxygen tension. Of the 14 isolations of *Hemophilus* which developed on both plates, it was not uncommon to observe growth appearing aerobically only in the vicinity of staphylococcus colonies (satellitism), whereas a large number of uniformly distributed colonies occurred on the corresponding anaerobic plate. This observation, although not seen in all isolations, was made frequently enough to raise some doubt as to whether satellitism depends entirely upon the elaboration of V factor. Since growth of certain strains resulting from the same inoculum may show satellitism aerobically and yet develop independently of staphylococci in the absence of oxygen, it is possible that a growth-stimulating substance, or substances, available only through the agency of staphylococci under aerobic conditions, is present in an assimilable form in the absence of oxygen.

Viridans streptococci seem less prone to develop anaerobically than do other types of streptococci or pneumococci. When growth does occur, "greening" is frequently suppressed so that the organism appears as a nonhemolytic streptococcus. Re-exposure to air usually results in subsequent discoloration.

*Semisolid Medium.*—A small proportion of the specimens were at the same time inoculated to a medium of the same composition (infusion), but to which had been added 0.3 per cent agar. The results were, as might be expected, intermediate between those of the aerobic and anaerobic plates. Nevertheless, in no instance in which the semisolid medium was positive, did the anaerobic plate fail to show growth. No medium especially designed for anaerobes, such as brain or cooked meat broth, was used. Any fluid or semisolid medium would have the same disadvantage that impelled us to restrict our further studies to the use of agar. A medium, such as Rosenow's brain broth, would undoubtedly permit the development of a large proportion of our "anaerobic" isolations. On the other hand, the separation and identification of a mixture



of bacteria would still require subcultures to aerobic, if not anaerobic, blood agar. The additional time and effort can be avoided by the direct inoculation of duplicate agar plates. Those laboratories, however, in which suitable anaerobic equipment is not available, might well use semisolid or meat media with subsequent subculture to aerobic blood agar. It should be pointed out that two disadvantages are inherent in this procedure. During incubation of a mixed culture in fluid media the more delicate pathogens may be completely overgrown or inhibited and, because of the necessary reinoculation to agar, additional time is required.

The addition of a reducing agent (0.03 per cent cysteine hydrochloride) to the medium was attempted in a small series of specimens with the thought that the presence of the cysteine would, perhaps, permit the growth of the anaerobic types of streptococci. This medium was quickly discontinued, however, because of the rapid and extensive production of "greening" about many different types of colonies, due, no doubt, to the formation of sulf-hemoglobin.

*Permanence of Anaerobic Requirements of Hemolytic Streptococci and Pneumococci.*—The hemolytic streptococci and pneumococci, with which we are here largely concerned, became, with few exceptions, quickly adapted to aerobic environment. In the large majority of instances the first transplant from anaerobic to aerobic blood agar resulted in varying amounts of growth. No effort was made to determine the exact proportion of permanently anaerobic hemolytic streptococci or pneumococci. Nevertheless, permanently anaerobic strains have been isolated without difficulty for experimental purposes. The occurrence of this type, however, would seem to be relatively uncommon.

#### DISCUSSION

The significant number of hemolytic streptococci and pneumococci isolated only by the use of anaerobic incubation would alone seem to justify the adoption of routine anaerobiosis in the clinical laboratory. In addition, the isolation of anaerobic nonhemolytic streptococci, actinomyces, *H. influenzae*, *Bacteroides* and *Clostridia* has suggested etiologic relationship to a number of unusual, and hitherto undiagnosed, cases. Indeed, it is our opinion that a satisfactory bacteriologic examination is possible only if an anaerobic culture is included. Soule (1932), in discussing the stimulus to the investigation of anaerobes resulting from the World War, says ". . . . a bacteriologic study of unknown material was not complete until the question of the presence of anaerobes was satisfactorily disposed of. Nevertheless, it is a matter of common occurrence to find that the knowledge reawakened by the war studies has lapsed and today the average complete bacteriologic examination of suspected materials consists of the isolation and attempted identification of the aerobic flora." We wholly agree with this opinion in so far as it applies to clinical bacteriology laboratories. It is our hope, therefore, that data herein presented may serve to emphasize the frequency with which anaerobic members of well-recognized aerobic, or facultative anaerobic, species are encountered in primary isolation.

The fact that such a large proportion of hemolytic or are stimulated by, lowered oxygen tension suggests that they be essentially anaerobic. In support of this hypothesis, the observation that most laboratory strains develop more readily in anaerobic media suggests that most laboratory strains develop more readily in anaerobic media. If these organisms are not fundamentally anaerobic, it is difficult to assume that the inclination toward anaerobic adaptation in the human body to an environment in which oxygen is low. Evans and Hartridge (1936) have written, "It is known that dyes such as methylene blue can often be reduced in the body. Thus, it would seem that normal tissue may have an inherent capacity and consequently one may readily imagine that debilitated tissue, especially with a diminution in the blood supply, may develop more anaerobic conditions. By the process of adaptation, anaerobic respiration may assume a prominent, and in some cases a characteristic character which must inevitably be reflected upon inoculation into anaerobic media. Under these conditions the surface of anaerobic blood agar plates shows conditions within the body more closely than does the surface of the plate. The fact that certain strains persist in their growth in anaerobic media leads one to the supposition that the period of adaptation to anaerobic conditions of considerable duration. There is no evidence to suggest that the anaerobic strains are more virulent than the aerobic strains."

By far the majority of anaerobic hemolytic streptococci have developed the capacity for growing aerobically on the surface of the agar. We have termed "temporarily anaerobic," Prévot<sup>6</sup> has termed "facultative anaerobes" to those organisms behaving in this manner. No attempt has been made, however, to group them on a taxonomic basis. Of practical significance is the fact that the development of anaerobes, such as deep tubes of blood agar, is not necessarily indicative of temporary anaerobes. Inoculation of a fastidious organism and bacterial antagonism can be overcome by the use of anaerobic blood agar will perhaps be a useful procedure.

A further comparative advantage of the anaerobic method is the tendency of anaerobic cultivation to inhibit the growth of many organisms. It is not infrequent, for instance, to observe a pure culture showing nonhemolytic staphylococci and *N. californicus* on a pure culture of pneumococci or streptococci on the anaerobic plate.

#### CONCLUSIONS

1. Approximately 2,500 routine, clinical specimens were inoculated upon aerobic and anaerobic blood agar plates.
2. The total number of specimens was divided in two groups, the first, beef infusion, and for the second, meat extract medium.
3. A percentage of 8.8 of the specimens of the meat extract medium were positive on the anaerobic plate but remained sterile on the aerobic plate. On meat extract medium, an incidence of 18.8 per cent of the specimens were positive on the anaerobic plate.
4. The most common organism appearing on primary anaerobic cultivation was the hemolytic streptococcus.

cocci were also frequently isolated. A possible explanation for the occurrence of these anaerobic types is discussed.

5. By far the great majority of these organisms quickly regained their ability to grow aerobically. A method is suggested for utilizing this adaptability.

6. A practical procedure for routine anaerobiosis in the clinical laboratory is recommended.

7. The occurrence of negative cultures from specimens showing a positive smear has been almost entirely eliminated.

8. Bacteriologic studies in the clinical laboratory are incomplete without anaerobic culture.

We wish to express our indebtedness to Dr. Ezra Casman, of the Abington Memorial Hospital, for his suggestions and helpful criticisms.

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## A SIMPLE METHOD FOR DETERMINING BACTERIAL REDUCTION OF NITRATES\*

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WASHINGTON, D. C.

THE routine method of testing bacterial cultures for nitrate reduction consists in inoculating broth containing potassium nitrate, and after incubation adding a reagent which produces a color in the presence of the nitrite radical. An alternative method consists in inoculating agar containing potassium nitrate and after incubation observing the culture for the presence of gas. A large amount of effort has been expended in devising a culture medium which would give consistent results in testing for nitrate reduction to nitrites.

Stickland<sup>1</sup> reported that the untreated cells of *E. coli* reduce nitrate to nitrite with great rapidity. We have made the same observation, and it occurred to us to extend the work to include different species of bacteria, and to compare the method to be described with the routine method.

### METHOD

The basis of the method consists in adding a solution of potassium nitrate to a suspension of the living bacterial cells to be tested. From fifteen minutes to one hour later the mixture can be tested for the presence of nitrites.

In our work we have used either twenty-four-hour broth cultures or some of the growth from an agar slant emulsified in saline. To 1 c.c. of culture, 0.2 c.c. of 1 per cent potassium nitrate is added. The mixture is shaken and allowed to stand for about one-half hour when it is tested for the presence of nitrite. If desired, quantitative determinations can be made at intervals and the rate of reduction determined.

We have tested 24 different species of bacteria using the routine method and the modified method, and we have found that there is complete agreement between the two. The following organisms reduced nitrates to nitrites by both methods: *Staph. albus*, *Staph. aureus*, *B. subtilis*, *B. anthracis*, *E. coli*, *A. cloacae*, *E. typhosa*, *S. paratyphi*, *S. schottmülleri*, *S. enteritidis*, *S. pullorum*, *S. gallinarum*, *Brucella abortus*, *Shigella paradysenteriae* (Flexner), *Shigella paradysenteriae* (Sonne), and *C. hofmannii*. The following organisms failed to reduce nitrates by either method: *Shigella dysenteriae*, *Alcaligenes faecalis*, *Vibrio metchnikovii*, *Strep. faecalis*, *Strep. hemolyticus*, *Strep. viridans*, *C. diphtheriae*, and *C. xerosis*.

### DISCUSSION

The simple method described has several advantages. A special medium is not required. Organisms difficult to grow in liquid media may be grown on

\*From the Department of Bacteriology, Georgetown Medical School, Washington, D. C.  
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enriched solid media, and the emulsified growth used in the test. Since the rate of reduction is increased if the concentration of organisms is increased, bacteria which grow sparsely may be concentrated by centrifugation and tested. With this method organisms which break down the nitrate radical to nitrite and then utilize the nitrite, and which would be recorded as negative by the routine method, will very likely be positive with the modified method. Old cultures in which most of the bacteria are dead will not reduce the nitrate. Media prepared with filtered material should not be used in growing bacteria to be tested, as Berkefeld and Seitz filters contain nitrite.

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### A RAPID STAINING METHOD FOR OPSONOCYTOPHAGOCYTTIC INDICES\*

MACKAY FLINN, NEW ORLEANS, LA.

WHILE working on a problem in which phagocytosis was concerned, we<sup>1</sup> developed a stain which we found very efficient. In view of the great amount of work now being done on the opsonocytophagocytic index in *Brucella* infections, it is likely that this method may find a field of usefulness.

The stain is made by adding 0.15 gm. methyl green and 0.5 gm. pyronine to 15 c.c. of 95 per cent alcohol. It is best mixed in a mortar. Eighty-five cubic centimeters of 3 per cent carbolic water is added slowly until the stain is completely dissolved. The completed mixture should stand at least a week with occasional shaking before it is used. Smears are made in the usual manner, dried in air, and flooded with the stain. They are allowed to stain four minutes, washed in tap water, dried in air, and examined. The cytoplasm of the white cells stains a faint pink, the nucleus a light red, and the organisms a deep red. The granules in the cytoplasm do not stain, and it is this feature which so much facilitates the bacterial count. When the usual blood stains are used, there is always some confusion between bacteria and cytoplasmic granules.

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\*From the Department of Pathology, Tulane University School of Medicine, New Orleans.  
Received for publication, May 3, 1939.

# A SLIDE FLOCCULATION TEST FOR THE DIAGNOSIS OF SYPHILIS\*

## PRELIMINARY REPORT

S. L. LEIBOFF, PH.D., M.A., NEW YORK, N. Y.

THE serologic diagnosis of syphilis is based upon two types of reaction, viz., the complement fixation phenomenon discovered by Bordet and Gengou<sup>1</sup> and adapted by Wassermann, Neisser, and Bruck<sup>2</sup> for the diagnosis of syphilis; and the precipitin reaction observed by Porges and Myer<sup>3</sup> and adapted for the diagnosis of syphilis by Michaelis.<sup>4</sup> Most likely both reactions are similar in nature in that they both depend upon the formation of a precipitate by the union of the "reagin" present in the serum globulin, with the lipoidal colloids of the antigen, and the subsequent adsorption of the complement by this precipitate. This theory is supported by the fact that examination of Wassermann reaction mixtures of syphilitic serum and antigen by the ultramicroscope shows the presence of precipitates even though these precipitates are invisible macroscopically.<sup>5</sup> The difference between the complement fixation and precipitin reactions seems to lie in the physical conditions which determine the size of the particles in the precipitates.

A great deal of attention has been paid to the development of suitable precipitin tests because these tests are speedier, easy to perform, and require fewer reagents. The most important advance in this field was made by Kahn<sup>6</sup> who produced a visible precipitate in syphilitic serum by using highly concentrated and sensitive antigen suspensions. To further enhance the visibility of the Kahn precipitate the centrifugation and resuspension of the precipitate in saline were suggested.<sup>7</sup> The foundation of the Kahn test is based upon four factors: namely, optimum concentration of ingredients, great instability of the antigen-saline suspension, quantitative relationship between antigen suspension and serum, and agitation of the ingredients. On the basis of these principles precipitin tests were devised by Kline,<sup>8</sup> Eagle,<sup>9</sup> Laughlin,<sup>10</sup> and others.

The slide flocculation test described in this paper has the following advantages: the antigen is stable and highly sensitive to syphilitic serum, the quantitative relationship between antigen and saline, and between antigen-saline suspension and serum, need not follow the strictest measurements but may be varied within certain broad limits without affecting materially the efficacy of the test. Other advantages are speed, easy manipulation, and good visibility.

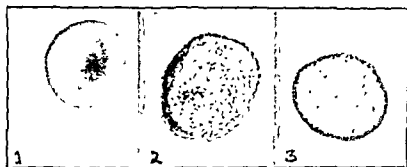
*Preparation of the Antigen.*—Fresh beef heart is used. Because of qualitative and quantitative variations in the lipoidal content of hearts obtained from different animals, at least six hearts should be used. After removal of fat

\*From the Laboratory of Lebanon Hospital, New York.  
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and other adventitious matter, the muscular tissue of the hearts is cut up into small pieces and passed through a meat grinder a few times until a very fine paste is obtained. The paste is spread in very thin layers over cheese cloth stretched tightly on wooden frames and dried continuously for two days at room temperature by means of an air blast. The dry tissue is broken up into small pieces and ground to a very fine powder. One hundred grams of the dry powder are shaken continuously in a liter flask with 400 c.c. of pure anesthesia ether for ten minutes and filtered through a fat-free filter paper. The powder on the filter paper is pressed lightly with a spatula until no more ether passes through the funnel. The moist powder is returned to the flask and again extracted with 400 c.c. of ether, following the same procedure as in the first extraction. This is repeated once more, making three extractions in all, with a total volume of 1,200 c.c. of ether. After the third extraction the moist powder is spread on a glass plate or on a clean paper and dried at room temperature until the odor of ether is completely gone. A weighed quantity of the ether-extracted powder is introduced into a dry flask, and absolute alcohol is added in the proportion of 5 c.c. of alcohol to 1 gm. of powder. The flask is covered tightly with a cork wrapped in tin foil and allowed to stand in a dark place at room temperature for four days. It is shaken daily for five minutes. At the end of the fourth day it is filtered through a double layer of fat-free filter paper and is cholesterinized with 6 mg. of pure cholesterol per cubic centimeter of extract. When the cholesterol is completely dissolved, it is allowed to stand at room temperature for twenty-four hours and filtered. About one-tenth volume of the alcoholic extract is transferred to a small flask and shaken vigorously with small amounts of dimethylamidoazobenzol until a saturated solution is obtained as evidenced by an excess of the dimethylamidoazobenzol. This is filtered through a double layer of fat-free filter paper into a small graduated cylinder, and three volumes of the alcoholic extract are added to the cylinder and mixed well. The rest of the alcoholic extract is saturated with sudan III dye and filtered. The two solutions are now mixed in the proportions of 85 volumes of the sudan III extract to 15 volumes of the dimethylamidoazobenzol and shaken well. The antigen is now ready for use. Antigens thus prepared eighteen months ago were found to contain their full potency.

*Performance of the Test.*—An antigen-saline suspension is prepared by placing a measured volume of antigen in a wide test tube, about 15 mm. outside diameter, and adding rapidly to it two volumes of 0.9 per cent sodium chloride solution. The mouth of the test tube is closed with a clean cork covered with tin foil, and the mixture is shaken rapidly with an up-and-down motion for one minute. The suspension may be used immediately and will not lose its potency for at least twenty-four hours. The serum is inactivated in the usual manner at 56° C. for half an hour. Two drops of inactivated serum (approximately 0.08 c.c.) are placed on a clean smooth glass slide, and one drop (approximately 0.03 c.c.) of the antigen suspension is dropped directly on the serum from a distance of about 1 cm. The serum and antigen are mixed together well by means of a thin glass rod, thereby spreading out the mixture

to form a circle about 15 mm. in diameter. The slide is now rotated with a gyratory motion for three minutes. In strongly positive sera large red floccules appear within a few seconds; after a minute or two of rotation, the floccules accumulate at the edges of the circle, leaving a clear colorless medium in the center. In weakly positive sera flocculation appears more slowly, in from one to three minutes, depending upon the amount of syphilitic reagin in the serum, and the floccules are of a smaller size and fewer in number. With a little experience, one soon learns to judge the strength of a



Figs. 1, 2, and 3.—Gross appearance (as seen with the naked eye) 1. Negative; 2. and 3. Positive.

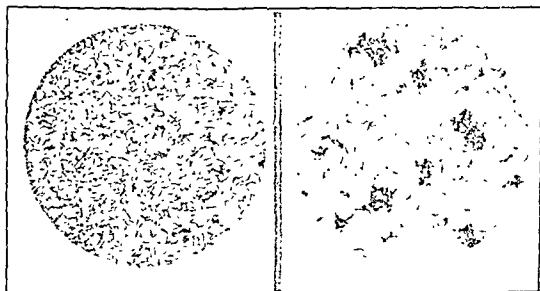


Fig. 4.

Fig. 5.

Figs. 4 and 5.—Microscopic appearance (under low power  $\times 320$ ). 4. Negative; 5. Positive.

positive reaction by the number and size of the floccules and by the speed with which they appear. Nonsyphilitic serum shows no flocculation whatsoever, and the edges of the circle remain smooth. When examined under the low-power lens of the microscope, negative serum shows a large number of slender needle-shaped crystals, somewhat thickened in the center and pointed at both ends. These crystals are evenly distributed throughout the microscopic field. Syphilitic serum shows large clumps of crystals surrounded by clear spaces.

Close to 10,000 tests were performed by this procedure during the last year and a half and compared with the Wassermann and Kahn tests. Good agreements were obtained with both the Kahn and the Wassermann tests. Particularly good agreements were obtained with 2,000 Wassermann tests performed with a highly specific cholesterinized antigen obtained through the courtesy of the New York State Health Department. The Wassermann tests performed with that antigen checked well with the clinical histories.



Because of these encouraging results, this simple and rapid method is recommended to the syphilologist in the hope that it may prove a valuable aid to him in the diagnosis and treatment of syphilis.

I wish to express my gratitude to Dr. J. C. Ehrlich, Director of Laboratories, Leiber Hospital, for his invaluable help and encouragement in this work.

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### AN IMPROVED TEST TUBE RACK\*

ROBERT F. ERVIN, M.S., NOTRE DAME, IND.

THERE is need for a test tube rack which will hold test tubes so firmly that they will not rattle nor slip out. The test tube rack described here was designed to eliminate some of the objectionable features found in most racks. Tubes are usually supported in holes punched in plates of metal or wood. Application of this principle makes it impossible to design a proper rack which will hold a variety of tubes tightly. Moreover, most metal racks and basket arrangements are easily damaged by bending and corrosion, and are difficult to repair. More than this, if a rack is to be used for tubes of varying sizes, only the tubes of uniform diameter will be held securely; all other tubes, unless supported at several points, will rattle and slip out.

It is felt that these disadvantages have been overcome in the rack illustrated in Fig. 1. The design consists primarily of a zigzag bar, supported at each end by uprights from a flat base. The entire construction is cast in one piece from white metal alloy, and is cadmium plated. A phosphor bronze coil spring is run down each side of the rack through holes in the apex of each V in the zigzag bar. These springs are fastened securely at each end by metal tapping screws. The standard model rack is  $12\frac{3}{4}$  inches long, 2 inches wide at the base, and  $2\frac{1}{2}$  inches high. It will accommodate tubes of  $\frac{3}{8}$  inch to  $\frac{3}{4}$  inch outside diameter, and has a capacity of 20 tubes.

\*From the Laboratories of Bacteriology, University of Notre Dame, Notre Dame.  
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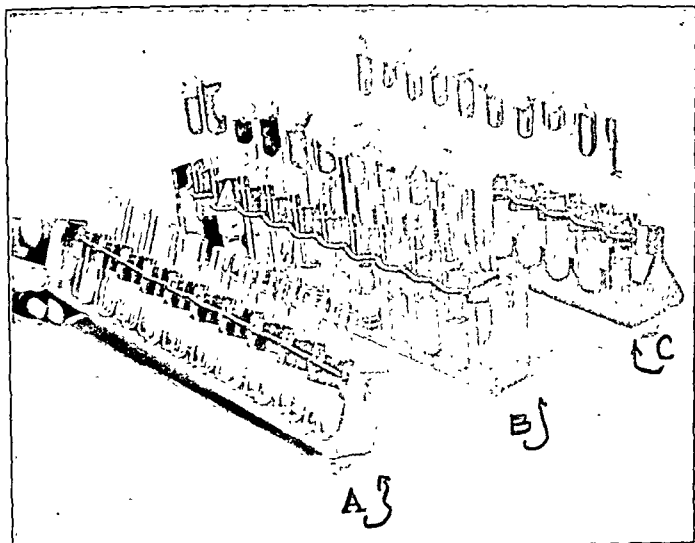


Fig. 1.—A. Clinical model (for  $\frac{1}{4}$  inch to  $\frac{3}{8}$  inch O.D. tubes). B. Clinical model (for  $\frac{3}{8}$  inch to  $\frac{1}{2}$  inch O.D. tubes). C. Standard model (for  $\frac{1}{2}$  inch to  $\frac{3}{4}$  inch O.D. tubes).

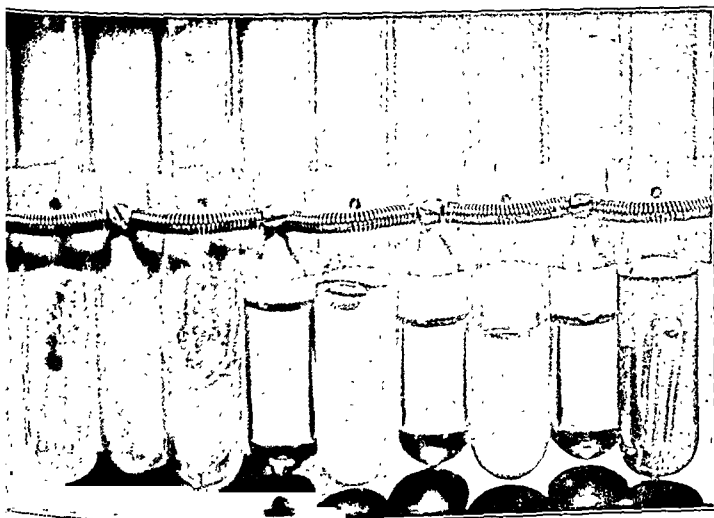


Fig. 2.—All tubes are visible from one position.

When in use, the tubes are held firmly in the V-shaped slots by the coil spring and rest against the base of the rack. They are easily slipped in and out of the slots, but are held so tightly in place that the rack may be turned upside down without causing the tubes to fall out.

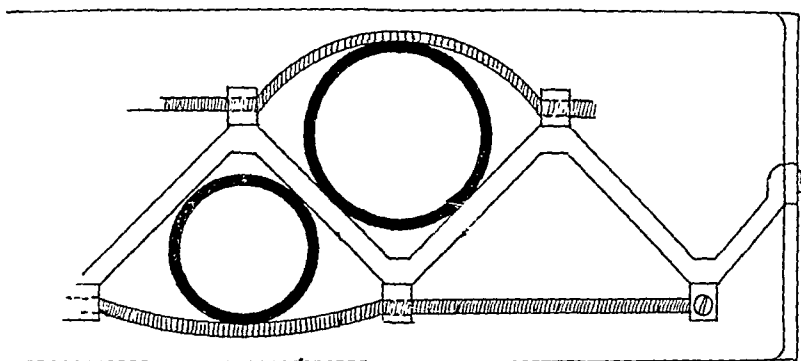


Fig. 3.—Showing three point support and continuous spring detail of the test tube rack.

The simple, sturdy construction makes cleaning easy and provides a rack which will endure hard use in a laboratory for years. The use of the zigzag bar makes all tubes visible from one position without removing any of them from the rack. This is a decided advantage when it is necessary to compare reaction-tubes with controls. The manner of holding the tubes allows the rack to be shaken in a shaker or by hand without rattle or danger of slipping. The design of the rack as well as the space-saving way of holding the tubes guarantees its efficiency for use in water baths, incubators, ice chests, etc.

In summary, a new test tube rack has been designed, and its efficiency has been proved in the laboratory. Its advantages are simplicity of design, ease of use, sturdiness of construction, economy of space, and rigidity as a support for test tubes.

This test tube rack is manufactured and distributed by Rayno Research, 3806 N. Ashland Ave., Chicago, Ill. After the design of Professor James A. Reyniers.

## AN ALL-GLASS ADJUSTABLE MOUSE CAGE\*

THOMAS W. RAY, PH.D., MILWAUKEE, WIS.

IT BECAME necessary to develop an all-glass adjustable mouse cage of sufficient size to hold a considerable number of animals. This type of cage is convenient to use and embodies the following special features: (1) ample ventilation, (2) glass wheels for exercising, (3) an attached drinking fountain, (4) ease in disassembling completely or in sections for cleaning, (5) convertible into one, two, three, or four units.



Fig. 1.

In a board  $1\frac{1}{2}$  inches thick, 12 inches wide, and 28 inches long, a groove  $\frac{1}{4}$  inch wide and  $\frac{1}{2}$  inch deep was made parallel with, and 1 inch from, the back, the full length of the board. At right angles to this groove, five similar ones were made, each 6 inches apart and 9 inches long, dividing the board into four 9 by 6 inch sections.

Plates of double strength glass, 9 inches by 9 inches, placed on edge in the grooves, form the back, end walls, and the partitions, while glass tubes

\*From the Department of Biochemistry, Marquette University Medical School, Milwaukee.  
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8 mm. in diameter cemented in holes bored 4 mm. apart through the board, and parallel with the edge, serve as the front and allow for ventilation. Suitable braces support the back and ends from the outside, while the middle partitions keep the back plates from tilting forward. The four sections are covered by plates, 6 inches by 12 inches, whose edges fit into grooves in the back braces. These top plates slope slightly toward the front, resting on the ends of the glass tubes and fitting between the end walls and the middle partitions, holding them erect. The front edges of the tops slip into a groove in a narrow board and are held down and back by latches securing the ends of the board to the cage.

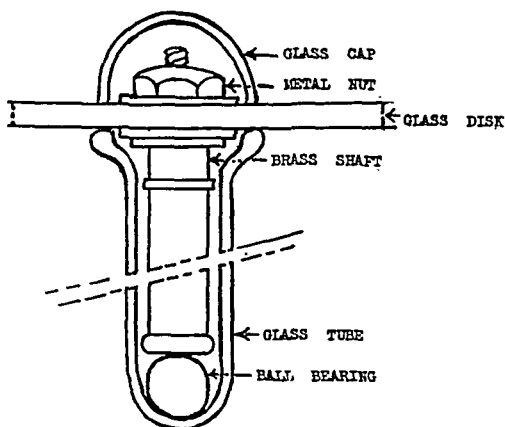


Fig. 2.—Exercise wheel.



Fig. 3.—Drinking fountain.

In the corners of each of the four sections holes,  $\frac{1}{4}$  inch in diameter and  $\frac{1}{2}$  inch deep, are made in the board. Glass tubes placed in these holes and extending 1 inch above the board serve as supports for the glass floors.

A glass disc,  $5\frac{1}{2}$  inches in diameter, frosted on the top side, to which is fitted a brass shaft, provides an exercise wheel. The lower end of the shaft rests on a steel ball in the bottom of a glass tube. The metal nut above the disk, and securing the disk to the shaft, is covered with a glass cap, while the shaft is completely protected by the glass tube. The wheel is held in position for use by inserting the shaft, covered by the glass tube, into a hole bored at a slight angle through the glass floor and into the board below.

Any one, or all, of the plates dividing the cage into sections may be removed, and plates, which extend only 2 inches above the glass floor, may be substituted, thus making it possible to convert the cage into one, two, three, or four units as desired.

The mice may be confined in any one small section while the other parts of the cage are being cleaned. A glass drinking tube is held erect and secure by inserting it into a glass cylinder and slipping the cylinder over two of the glass tubes forming the front of the cage. A short cylinder is first passed over the two tubes holding the mouth of the drinking fountain the desired height above the floor.

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## A NEW SAHLI TYPE HEMOGLOBINOMETER\*

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RUSSELL L. HADEN, M.D., CLEVELAND, OHIO

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GOWERS first suggested in 1878<sup>1</sup> the quantitative estimation of hemoglobin by diluting a fixed amount of blood in a graduated tube until the color matched that of a standard in a tube of equal diameter. The blood was diluted with distilled water and compared with a solution of picrocarmine in glycerin. A few years later Sahli<sup>2</sup> modified this method by converting the blood into acid hematin by mixing with N/10 hydrochloric acid and comparing it with a solution of acid hematin. Haldane<sup>3</sup> still later used a carbon monoxide-hemoglobin standard and converted the hemoglobin of the unknown blood into this compound by bubbling illuminating gas through it.

The Sahli method of hemoglobin determination has long been a favorite one. The apparatus is inexpensive, and the brown color of hematin is easily matched. It is necessary that the bore of the standard and the graduated tube be exactly the same, the pipette must be accurate, and the standard must be correct. The liquid standards of acid hematin were soon found unsatisfactory because of fading and other difficulties and have been replaced by glass standards to match acid hematin. The round tube has also been replaced by a square one which is made over a metal core and so has a uniform inner diameter.

Even after the square tube and an accurate glass standard which matches acid hematin closely are provided, one difficulty with the Sahli method remains. The glass standard is the same throughout its length. The blood is gradually diluted until it matches the standard. It is very difficult to tell when this match is correct or to choose among several approximate matches. Usually the dilution is continued until the unknown is a lighter shade than the standard.

A new Sahli apparatus has been devised to overcome these objections (Fig. 1). The mechanical features have been developed by C. A. Hausser and Son; I have standardized the apparatus. The glass standard is divided into three windows, separated by opaque bands (Figs. 2 and 3) with a 5 per cent variation in the hemoglobin value between each of the three standards. The central window is the standard color, with which the specimen is to be matched (Fig. 4). The lower window is 5 per cent darker, and the upper window is 5 per cent lighter. There are thus always three standards for comparison. When

\*From the Cleveland Clinic, Cleveland.

The Sahli-Haden hemoglobinometer is made by C. A. Hausser and Son and is sold by the Arthur H. Thomas Co., West Washington Square, Philadelphia, Pa.

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the correct reading is made, this match is evident from the lighter color above and the darker color below. The colored glass provides a close match for the acid hematin, as shown by spectrophotometric readings.

The color in the graduated tube and that of the glass standard are brought into juxtaposition with a pair of prisms which locate the two color factors in the same focal plane. A square standard tube (Fig. 5) is employed. A magnifier is used to enable focusing past the surface of the calibrated tube and the plane glass surface of the colored glass standard. The inside diameter of the glass tube and the glass standard are checked photoelectrically. An Ulrich pipette (Fig. 6) is used in place of the original Gowers pipette employed with the Sahli apparatus.

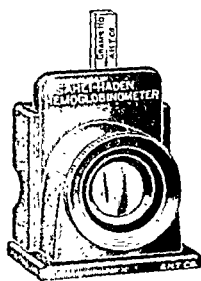


Fig. 1.

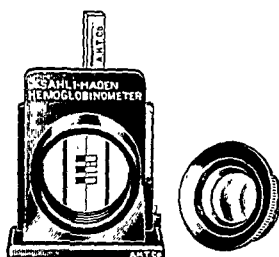


Fig. 2.

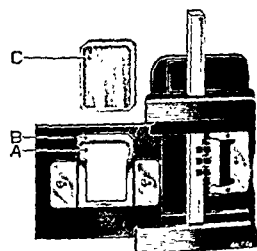


Fig. 3.

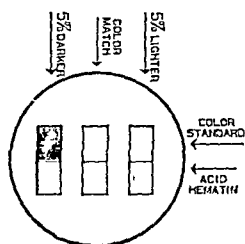


Fig. 4.



Fig. 5.

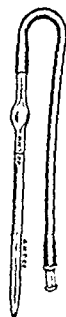


Fig. 6.

Fig. 1.—Front view with magnifier in position.

Fig. 2.—Front view with magnifier removed, showing color matching rectangles behind glass prisms.

Fig. 3.—Rear view with ground glass or "daylite" plate removed, showing one-half of color matching rectangles.

Fig. 4.—Field of view showing both halves of the three color matching rectangles.

Fig. 5.—Graduated glass tube of square cross section for the acid hematin solution.

Fig. 6.—Ulrich pipette for collecting blood specimens.

**Calibration.**—The apparatus has been standardized to read in grams of hemoglobin per 100 c.c., by comparing readings with those made on the Haden-Hausser laboratory model hemoglobinometer. No direct comparison has been made with the iron-content and oxygen-content methods, but the Haden-Hausser instrument has been repeatedly proved to give results comparable to those obtained by these two methods.<sup>4</sup>

The comparative readings with twenty different specimens of blood on the two instruments are given in Table I.

## PROCEDURE

The Ulrich pipette must be thoroughly dried. Blood is drawn to the 20 c.mm. mark. The tip of the pipette is wiped off, and the bulb is filled with N/10 hydrochloric acid. This is then completely emptied into the graduated tube. If an ordinary 20 mm. pipette is used, N/10 hydrochloric acid is run into the graduated tube in the two graduations, blood is drawn to the graduation mark on the pipette and run into the acid in the tube. The diluted blood should be drawn back into the pipette several times to completely empty it.

TABLE I

SPECIMEN NO.	HEMOGLOBIN IN GRAMS PER 100 C.C. BY	
	HADEN-HAUSSEY LABORATORY MODEL HEMOGLOBINOMETER	NEW SAHLI-HADEN HEMOGLOBINOMETER
	<i>gm.</i>	<i>gm.</i>
1	16.0	16.0
2	12.0	11.8
3	9.5	9.1
4	11.0	10.8
5	16.0	16.0
6	14.0	13.8
7	16.0	16.2
8	8.1	8.2
9	16.0	15.8
10	10.5	10.5
11	8.0	7.5
12	9.0	9.0
13	16.0	16.2
14	10.7	10.7
15	8.0	8.0
16	8.0	7.8
17	11.0	10.6
18	8.0	8.2
19	10.5	10.8
20	8.0	8.2
Mean	11.32	11.28

After standing thirty minutes, the color comparison is made. If the reading is made after ten minutes, 4 per cent is added; after fifteen minutes, 3 per cent; and after twenty minutes, 2 per cent. Readings can be made either with daylight, using the ground glass back plate, or with artificial light using a "daylite" glass plate.

In making the color comparison, the magnifier should be focused sharply on the windows of the black mask. Add water with an eye dropper, mixing constantly with the stirring rod until the color of the specimen approximates that of the lowest window. Then add water carefully until the specimen matches the middle window. If the match is correct, the lower window will be darker and the upper window lighter than the specimen.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**PREGNANCY.** An Investigation of the Patterson Test for the Chemical Diagnosis of Pregnancy, Krieger, V. I. M. J. Australia 1: 494, 1939.

The author concludes that the Patterson test is the most satisfactory chemical test so far advanced for the detection of early pregnancy.

The Patterson test is carried out in the following way:

Fifty cubic centimeters of concentrated early morning urine, just alkaline to methyl red, are heavily inoculated with *B. coli* and incubated overnight. While the urine is still warm, 0.5 gm. of sodium bisulfite is added, dissolved by being shaken, and allowed to stand for fifteen minutes. The urine is extracted twice with 40 c.c. volumes of ether. The combined ether extracts are washed with water and then shaken with 30 c.c. portions of 10 per cent sodium carbonate until the carbonate layer is quite colorless. The ethereal solution is again washed with distilled water. The ether layer is extracted with two 30 c.c. volumes of N/10 sodium hydroxide. The combined alkali solutions are acidified to Congo red with a 25 per cent solution of sulfuric acid and then extracted with two 40 c.c. volumes of ether. These combined ether extracts are washed with distilled water, with 10 per cent sodium carbonate, and finally with two volumes of distilled water. The final clear, colorless, ether solution is transferred in two portions to a 50 c.c. flask, and the ether is removed by distillation in a water bath at 70° C. The final traces of moisture are removed by application of direct suction to the flask. The dry residue is heated for ten minutes in a boiling water bath with 1 c.c. of phenolsulfonic reagent (9 c.c. of pure carboic acid and 14 c.c. of concentrated sulfuric acid). The mixture is cooled under the tap, treated with 1 c.c. of a 5 per cent solution of sulfuric acid, and is then reheated in a boiling water bath for two and a half minutes. A positive reaction is indicated by a pink or red color; in the absence of this change, the reading is negative.

**ERYTHROCYTE SEDIMENTATION,** Practical Value in the Management of Pulmonary Tuberculosis, De Cecio, T., and Elwood, B. J. Am. Rev. Tuberc. 39: 748, 1939.

The study of the sedimentation rate with a view to establishing its practical value in the management of tuberculosis of the lungs reveals the following facts:

In the initial study of a case, the occurrence of an elevated rate indicates, in a considerable majority of instances, the presence of an active lesion. However, the presence of a normal rate does not exclude an active lesion.

In the correlation of serial rates with definite pathologic trends, the case percentage (55 per cent) of compliance of the rates with the anatomic course is not of sufficient significance to be of practical value.

No greater percentage of relapse or reactivation occurred in the group discharged with elevated rates than in the one with normal rates. Furthermore, a considerable majority of those patients with sustained elevation of the sedimentation rate have remained well from one to five years.

The information obtained from the clinicopathologic study reveals not only all that also more information than can be gleaned from sedimentation rates alone, and with a greater degree of accuracy.

Thus the use of the sedimentation rate in the management of pulmonary tuberculosis as a criterion of activity, course, and prognosis is not of sufficient clinical value to be essential in the care of the tuberculous individual.

**BALANTIDIASIS**, Young, M. D. J. A. M. A. 113: 1939.

Seven infections with *Balantidium coli* in white women were observed by the author in South Carolina and 2 cases were found in Tennessee by Meleney.

The infected patients had chronic diarrhea and occasionally dysentery. *Trichuris trichiura*, *Necator americanus*, and *Strongyloides stercoralis* were present also, and consequently the symptoms may not have been due entirely to the balantidia.

Frequent examinations of the stools were made in cases of infection. Trophozoites were found 127 times in 145 examinations, a percentage of 87.5. Cysts were found 13 times, a percentage of 8.9.

Epidemiologic evidence indicates that the present infections were contracted from human sources rather than from hogs; swine are generally thought to be the source for most infections in man.

The balantidia disappeared from the stools of two patients treated with carbaisone. The stools of one patient were still free after five months. One month had elapsed since the treatment of the second and no balantidia had been seen. Oil of chenopodium given orally was not efficacious.

Few reports of this disease occur in the literature. Previous to this report, 32 cases had been reported from 16 states. This report adds 9 more cases from 2 states.

The evidence indicates that the disease may be more prevalent than is reported.

**ENDOCARDITIS**, Subacute Bacterial, Evaluation of Sulphanilamide in the Treatment of, Spink, W. W., and Crago, F. H. Arch. Int. Med. 64: 228, 1939.

Sulfanilamide was administered to 12 patients with subacute bacterial endocarditis. The etiologic agent was *Str. viridans* in 11 patients and *Staph. albus* in one. Of the 2 patients who appeared definitely improved after receiving the drug, one eventually died of the disease, and at the time of writing the other has been well for nine months.

All the patients had bacteriemia. Sulfanilamide rendered the blood stream sterile in 6 of the 12 patients. This bactericidal effect appeared to be only temporary, except in 2 patients.

In 4 patients there was a decline in the temperature coincident with the administration of sulfanilamide. There did not appear to be any relationship between the degree of fever and the therapeutic effect of sulfanilamide.

Although the drug was taken for a long period by several patients, there was a marked decline in the erythrocyte level in only one person. In no patient was a depression of the leucocytes observed.

There was no definite relationship between the amount of free sulfanilamide in the blood and the effect of the drug on the bacteriemia.

The use of sulfanilamide in the treatment of patients with subacute bacterial endocarditis is of doubtful value because of the nature of the focus of infection.

It appears from the foregoing observations that the administration of sulfanilamide to patients with subacute bacterial endocarditis will, in some instances, render the circulating blood free of organisms. Except for 2 patients this bactericidal effect was only temporary and depended on the continued use of the drug. In 2 patients definite improvement followed the use of sulfanilamide. However, the authors believe that sulfanilamide and its related compounds will be of doubtful value in the treatment of patients with subacute bacterial endocarditis because of the very nature of the focus of infection. The proliferating mass of bacteria situated well beneath the surface of the vegetation is probably protected, at least in part, from the action of free sulfanilamide in the blood, as well as of specific antibodies. When the organisms approach the surface of the vegetation, they are carried off in the circulating blood, and under these circumstances may be killed. An analogous therapeutic situation is now recognized in the treatment of patients with bacteriemia due to beta hemolytic streptococci. Lockwood stated that in a number of instances of infection of the blood stream an infected thrombus in a large vessel has prevented satisfactory elimination of the bacteriemia.

Since the presence of sulfanilamide in the blood may have a bacteriostatic effect on some strains of *Str. viridans*, sulfanilamide probably should be administered to any patient with valvular lesions who may be subjected to oral surgical procedures. It is well known that after the extraction of teeth or after a tonsillectomy temporary bacteremia with *Str. viridans* may result.

**CHEMOTHERAPY, Present Position of, by Drugs of the Sulphanilamide Group, Browning.**  
C. H. Brit. M. J., Aug. 5, 1939, p. 266.

While cure of certain experimental general infections with bacteria by sulfanilamide compounds is an enormous advance, the efficacy of the present drugs is by no means ideal. So far at least as mice are concerned, sulfanilamide is surpassed by, for example, M & B 693, rodilone, and especially the related monoacetyl-p-p-diaminodiphenyl-sulfone. But with them all the range of effective dosage is not wide, and treatment to be most successful must be begun early.

Further work on the absorption and excretion of members of the series, and on the chemical transformations which they may undergo in the body, as well as clinical observation, is required in order to enable them to be used to the best advantage as regards the choice of drug, and the dosage in amount and spacing in time. For instance, in treating infection of the meninges by oral or subcutaneous administration it will probably be found that one which rapidly attains a high concentration in the cerebro-spinal fluid, such as sulfanilamide, ought to be given in the first place, while more potent but more slowly absorbed compounds, provided they ultimately reach the spinal fluid in sufficient amount, are used to re-enforce and maintain the action.

Where conditions favorable to general infection have arisen, prophylactic use of the sulfanilamide group should be valuable. Where infection has been established, the best therapeutic effects are secured in acute, diffuse conditions, without marked local tissue changes. Hence these drugs are not likely to obviate operative procedures where focal lesions, such as accumulations of pus or extensive necrosis of bone or other tissues, have occurred. Accordingly, powerful antiseptics which act when brought into close contact with the organisms locally, and which are relatively harmless to the tissues, will continue to be required also in the treatment of many septic infections.

**SICKLE-CELL ANEMIA, Sedimentation Rates of Sickled and Non-Sickled Cells in**  
Bunting, H. Am. J. M. Sc. 198: 191, 1939.

Sickled erythrocytes from patients with sickle-cell anemia and the sickle-cell trait did not form rouleaux and remained almost unsedimented after one hour's time, while nonsickled cells from the same patients formed rouleaux and sedimented.

**BIOLOGICAL FLUIDS, Preservation of, Maier, E. J. Bact. 38: 33, 1939.**

A new compound, alkyl-dimethyl-benzyl-ammonium chloride, has been studied in reference to its germicidal, bacteriostatic, and inactive zones in accordance with the term "disinfectant spectrum," as proposed by Marshall and Hrenoff (1937). The effect of this compound in dilutions of 1:4,000 has been studied in the cornea of the rabbit by histologic methods. Comparisons were made with 3.5 per cent tincture of iodine. It was shown that one single application of the iodine tincture was sufficient to destroy the cornea completely and to produce permanent opaqueness in the same. The compound under discussion has been applied to the cornea daily for ten days, with no consequences.

A dilution of 1:50,000 in staphylococcal bacteriophage was found not to interfere with the reproduction of the phage. Furthermore, the phage-disinfectant mixture was found to be capable of reproduction of new phage after three months' contact in the refrigerator. The same concentration of the compound was employed in the preservation of vaccines and venom solutions.

**PNEUMONIA, Pneumococcic, Treatment of, with Concentrated Antipneumococcic Rabbit Serum, Wood, W. B., Jr. J. A. M. A., 113: 745, 1939.**

Fifty patients with pneumococcic pneumonia of nine different types were treated with concentrated antipneumococcus rabbit serum. Eight patients with type III pneumonia were treated and 4 died, the serum seeming to be relatively ineffective with this type. For the remaining 42 patients the fatality rate was 14.3 per cent, although 26 per cent of the patients had bacteremia and a like number had multilobar consolidation. Excluding the patients with type III pneumonia, all the patients who failed to survive had more than one lobe involved, and all had more than five colonies of pneumococci per cubic centimeter in the blood culture except one, an 82-year-old white woman who died of congestive heart failure and uremia. The incidence of empyema was 7 per cent. Thermal reactions were relatively common, occurring in 26 per cent of the patients, and delayed serum sickness developed in more than one-third of the patients.

**RABIES in Birmingham, Alabama, Denison, G. A., and Dowling, J. D. J. A. M. A. 113: 390, 1939.**

The data presented are not of a type to warrant definite conclusions. The following general statements are offered as implicit in the experience cited:

Rabies is primarily a veterinary problem. Regardless of its prevalence among dogs, it will probably never cause sufficient morbidity or mortality among human beings in this region to allow its classification as a public health problem of importance.

If rabies were as easily contracted by man as is commonly supposed, it would become one of the leading causes of death in Alabama. Instead, it continues to be a very rare disease.

In this region there appears to be little relationship between mortality from rabies and the administration of antirabies vaccine.

Aside from an actual bite or scratch, circumstances of exposure rarely warrant vaccine treatment.

When considering the advisability of treatment for exposures other than an actual bite, the possibilities of vaccine paralysis or other serious complications should be considered.

The problem of rabies, as it is known in Birmingham, appears to be one of home-owned dogs (pets) and not of unidentified strays.

**TUBERCULOSIS: Comparison of Tuberculin Patch Test with Mantoux Intracutaneous Test, Taylor, G. Am. Rev. Tuberc. 40: 236, 1939.**

A total of 25.7 per cent of 744 subjects gave a positive Mantoux and a positive patch test; 68.6 per cent gave a negative Mantoux and a negative patch test. Thus the two tests yielded comparable results in 94.3 per cent of the persons tested.

In 3.6 per cent a negative Mantoux and a positive patch test were observed; of these 27 subjects, 26 were found among young adults, students, and nurses.

In 2.0 per cent a negative patch test and a positive Mantoux were seen; of these 15 subjects, 12, or 80 per cent, were above 50 years of age.

In this series of 744 cases in which the Vollmer patch test was compared with the Mantoux intracutaneous test, using 0.1 mg. O.T., the results indicate that the simpler Vollmer test is equally as effective as the Mantoux test, and possesses many advantages for the physician as well as for the patient.

**TUBERCULOSIS: Culture Method in Sputum Examination, Loesch, J., and Petrik, F. Am. Rev. Tuberc. 40: 233, 1939.**

A total of 1,028 sputum examinations on the same number of clinic patients are reviewed.

When the culture method was used to supplement smear and concentration, 24 per cent of the total number of positive cases were detected by culture only.

The economic advantages of this method are self-evident.

It is suggested that all sputa which are negative on direct smear be cultured.

The cultural method used in this study is that described by Petrik (Am. J. Clin. Path. 8: 134, 1938).

#### **BROMIDE Intoxication, Gundry, L. P. J. A. M. A. 113: 466, 1939.**

Bromide intoxication is a common condition, and, judging from the hundreds of cases which have been reported, it is prevalent in all parts of this country. In spite of its prevalence, many physicians still fail to recognize this condition.

The cause of bromide intoxication is most commonly a physician's prescription written for an alcoholic addict, a psychoneurotic person, or a psychotic patient. If the chloride intake is low, or if the patient is suffering from some debilitating disease, intoxication occurs more readily.

The severity of the symptoms of bromism closely parallels the elevation of the blood bromide level. These symptoms are characteristic and are found in some degree in all patients with a blood bromide content above 150 mg. per 100 c.c.

An alert physician should be able to make the diagnosis from the history and physical examination. The blood bromide should confirm the diagnosis.

The prognosis should be guarded until the underlying condition has been studied.

Treatment of bromide intoxication is divided into preventive and curative measures. The latter is easy and satisfactory, but the former is more important for the physician to remember.

#### **SEDIMENTATION TEST, Choice of Technique for, Hambleton, A., and Christianson, R. A. Am. J. M. Sc. 198: 177, 1939.**

The data obtained at this tuberculosis sanatorium indicate that the sedimentation test remains today essentially what it was when Fahraeus introduced the test in 1918, and that all the significant clinical data which may be obtained by sedimentation procedures can be found by a single one-hour reading by the Westergren technique. Alleged improvements upon the Westergren sedimentation method have been presented which appear theoretically correct, e.g., correction for cell volume, use of heparin in place of citrate, and graphic methods of recording the results. Instead of making the test more valuable, these changes have the reverse effect, and either lead to results of less clinical value, or make the test more tedious to perform or less clear to interpret without increasing its clinical value. Hence it is recommended that the Westergren method, by reason of its simplicity, reliability, and priority, should be adopted as the standard method of performing the sedimentation test.

In conclusion, those who use the Westergren technique should keep in mind that, due to the narrow bore of the tube, it is all the more important to see that the tubes are perfectly vertical during the test.

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## *CLINICAL AND EXPERIMENTAL*

### INDUCED RETICULOCYTOSIS IN THE RAT AND ITS RELATION TO THE LIFE DURATION OF THE RED BLOOD CELL\*

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A REVIEW of the literature reveals that four different approaches have been made to the problem of the life span of the red blood cell. These may be grouped as follows: (1) bile pigment output,<sup>1, 2</sup> (2) agglutination of transfused cells,<sup>3, 7</sup> (3) reduced oxygen tension of the air,<sup>8</sup> and (4) blood replacement following hemorrhage.<sup>9</sup>

In no case have the different methods given comparable results. However, it must be recognized that in all these studies the number of subjects making up any given series was small. In one instance the report was made upon a single animal.<sup>9</sup> In the present study we have applied the principle of blood replacement following hemorrhage to the problem of the life duration of the red blood cell in the rat using daily reticulocyte counts as the index.

It became apparent immediately that a great deal of preliminary work must be done upon rats if we were to correctly translate our results into evidence bearing upon the life duration of the red blood cell of this animal. We have, therefore, included in this report also, a method for making permanent preparations of rat reticulocytes; a report upon the control level of reticulocytes in a total of 22 animals; and the reaction of animals to various degrees of single and repeated hemorrhage.

#### METHOD

It was found that constant successful staining of rat reticulocytes was best accomplished by the direct smear method, using brilliant cresyl blue stain. The slides are first stain processed. The blood smear is made upon a cool slide (60° to 70° F.) and promptly, while still wet, placed in a humidity chamber (70 to

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80 per cent saturation) for three minutes to permit staining. The whole procedure follows in detail.

There are three major steps: (1) preparation of the slides, (2) preparation of the blood smears, and (3) counting.

*Preparation of the Slides.—Part 1, Washing.* The slides are placed one by one into sulfuric acid-bichromate cleaning fluid for twenty-four hours. They are then removed and dropped one by one into running water and washed for three hours, then rinsed three times in distilled water and placed in 80 per cent alcohol until needed.

*Part 2, Stain Processing the Slides.* The slides are removed one by one from the 80 per cent alcohol and carefully dried with a piece of clean cheesecloth, previously washed with ivory soap, rinsed in distilled water, and dried. (It is convenient when preparing large numbers of slides to do them in groups of 15 or 20.) To proceed with the stain processing arrange the clean slides in a row from left to right in front of the operator, placing them at an angle of about 45°. Pour 5 c.c. of a saturated alcoholic (absolute) solution of brilliant cresyl blue into a 50 c.c. pyrex beaker and proceed promptly as follows: Pick up the first slide by the edges, holding it between the thumb and index finger. Dip the opposite end into the stain, touching the bottom of the beaker; remove immediately with its burden of stain\* and touch it to the top surface of the second slide, about 2 cm. from its near end. Now by supporting the slide *only* upon the tip of the middle finger at an angle of about 25°, push the stain-laden slide (No. 1) smoothly but rapidly across the slide whose surface is to be stained (No. 2). Then lay the slide across two glass rods to dry. (The speed at which the stain is spread determines the thickness of the stain film.) A thin even film of stain results. Thus each slide has been partially stain processed, No. 1 on its end; No. 2 on its top surface. Now in like manner use slide No. 2 to process the surface of slide No. 3; slide No. 3 to process the surface of slide No. 4, and so on, until the whole group has been treated. Finally, the last slide is used to apply the surface coat to the first slide, completing the task. These slides keep indefinitely, but must be cooled to about 60° F. before making the blood smears.

*Preparation of the Blood Smears.*—There are two parts: (1) the preparation of humidity chambers to prevent the smears from drying too quickly; and (2) the preparation of the smear, counterstaining, and mounting. Three simple, yet very satisfactory, humidity chambers may be quickly prepared by placing several layers of blotter paper in the bottom of a shallow baking pan. Moisten with tap water and invert three 10 cm. Petri dishes over the moistened paper. A weight may be placed upon each Petri dish to prevent too much leakage of the moist air. In a few minutes after the chambers are set up they are ready for use. The use of three chambers was found convenient and gives just about the right timing (two to three minutes) when a large number of smears are being made at one time.

Rotation through the series of chambers is accomplished by placing No. 1 blood smear under the first dish; make No. 2 and place under the second; make No. 3 and place under the third. By this time No. 1 slide can be removed for drying. Make No. 4 slide and put under the first dish. Remove No. 2 slide for drying, and so on, through the entire series.

\*The rate at which the slide is removed determines the amount of stain carried. Quick removal gives a heavily laden slide, and vice versa.

*Part 2. Preparing the Blood Smear* A small drop of fresh blood is touched to the stained surface of a cool (60° to 70° F.) slide. Immediately this slide is dropped conveniently in front of the operator at an angle of about 45°, and the stain-processed end of a second slide is touched to the first slide just in front of the drop of blood and immediately drawn backward to contact the blood which quickly spreads along the entire edge of the supported slide. As soon as this is observed, the slide again resting (as already described) upon the tip of the middle finger and at a 25° angle to the surface is slowly and smoothly pushed across the surface of the first slide, spreading the blood into a thin even film. (The rate at which the smear is spread determines the thickness of the film, i.e., a slowly spread film is thin, and vice versa.) Immediately place the wet blood film beneath the (Petri dish) humidity chamber for three minutes; remove; dry in the air, and counterstain with Wright's blood stain. Mount in balsam.

*Part 3. Counting.* To facilitate accurate counting of cells, the ocular of the microscope is provided with four cross hairs, dividing the field of observation into eight equal sectors.

The reticulocyte count is accomplished by first surveying the slide under low power to select a suitable area for counting. Practice will soon establish that these areas show an even distribution with the cells moderately separated.

TABLE I  
SHOWING SEX, AGE, AND WEIGHT OF ANIMAL MATERIAL

SERIES NUMBER	NUMBER OF LITTER MATES	AGE BEGINNING OF EXPERIMENT	MALE	AVERAGE WEIGHT	FEMALE	AVERAGE WEIGHT	DURATION OF EXPERIMENT
		Months		Grams		Grams	Days
1	4	13	1	348.2	3	204.3	91
2	8	3	4	196.5	4	138.1	55
3	10	4	4	264.7	6	188.5	91

The area located is then observed under oil immersion and usually found to contain from 200 to 300 red blood cells per field. The counting procedure is simple. Starting from a given point and counting adjacent fields, 1,000 red blood cells, including the reticulocytes, are counted in blocks of 100 cells each. The reticulocytes in each block of 100 red blood cells are recorded separately. This is done to give information on the reticulocyte distribution, which occasionally is sufficiently irregular to warrant recount. Once in a while it is necessary to count 2,000 red blood cells to establish the "reticulocyte value" of a slide. Upon completion of the count (at least 1,000 cells should be counted) the reticulocytes are summed up and expressed in per cent, or better still as reticulocytes per 1,000 red blood cells.

*Animal Material.*—Twenty-two rats (Wistar strain) divided into 3 series, representing 3 litters of our best stock, were used (see Table I). The animals were selected and placed in individual wire cages. They were always handled gently with bare hands. No sudden movements or noises were permitted during the experimental periods. All received the same stock diet and care.

#### PROCEDURE

The processed slides are brought into the animal room and placed beside the humidity chambers (already prepared, see Method). The animal is then



removed from its cage, and placed in a box 4 inches by 7 inches by 3 inches, provided with a sliding lid and a vertical slit  $\frac{1}{2}$  inch wide in the end section to accommodate the tail. The animal's tail is gently held in the left hand while a small piece of the tip of the tail is clipped off with a small sharp scissors. If quickly done the animal will not move. A small drop of blood will immediately appear; this is quickly touched to the stained surface of the cooled slide and spread. The slide is promptly placed under the Petri dish to complete the staining. After three minutes it is removed from the humidity chamber and allowed to dry in the air. The smears may be stored temporarily or counterstained and mounted at once.

TABLE II  
SHOWING RETICULOCYTE CONTROL VALUES FOR EACH ANIMAL

ANIMAL NUMBER	SEX	AVERAGE CONTROL RETICULOCYTE COUNT	CONTROL PERIOD	MAXIMUM RETICULOCYTE FLUCTUATION $\pm$ CONTROL AVERAGE
		Per 1,000 R.B.C.	Days	Per 1,000 R.B.C.
R-54	F	16.2	5	3.0
R-13	M	19.0	7	4.5
R-51	F	18.0	7	3.0
R-4	F	18.8	8	2.5
R-9	F	19.7	8	3.0
R-10	F	20.0	8	4.5
R-53	F	15.7	9	3.5
R-14	M	17.0	9	5.0
R-7	F	19.5	9	7.5
R-59	F	15.1	10	4.0
R-57	M	16.5	10	2.5
R-11	M	18.9	10	9.0
R-3	M	20.4	11	2.0
R-52	F	17.3	12	4.5
R-8	F	20.0	13	5.5
R-12	M	21.0	13	3.0
R-56	M	19.8	18	6.5
R-2	F	19.8	20	4.0
R-1	F	22.0	29	4.5
R-55	F	17.2	33	7.5
R-60	M	18.2	51	10.0
R-58	M	20.0	65	12.5

In this manner, daily reticulocyte counts were made on all animals throughout the experimental period. A period of from five to sixty-five days was consumed in establishing control levels before the animals were bled. Bleeding was accomplished by inserting a 26 gauge syringe needle into one of the lateral tail veins. A skin incision over the vein near the root of the tail simplified the procedure. Counts were continued daily after the bleeding, until the control reticulocyte level was again reached and maintained. In certain instances, explained later, the hemorrhage was repeated.

*Reticulocyte Level in Rats' Blood.*—In three series made up of 13 females and 9 males, a total of 365 control determinations were made over periods of from five to sixty-five days. The average control reticulocyte count for the group (22 animals) was 18.6 cells per thousand red blood cells (males, 19 reticulocytes per thousand; females, 18.3). The highest values for individual animals were 21 reticulocytes per thousand over a 13-day period for males, and 22 reticulocytes per thousand over a 29-day period for females. The lowest individ-

ual values over 10-day periods were: female, 15 reticulocytes per thousand, and male, 16.5. Normal fluctuation in the reticulocyte level is only moderate, and over long periods (65 days) did not exceed 25 reticulocytes per 1,000 red cells (Fig. 1). The average fluctuation for the group was found to be 10 cells per 1,000 red blood cells, or  $\pm 0.5$  (see Table II).

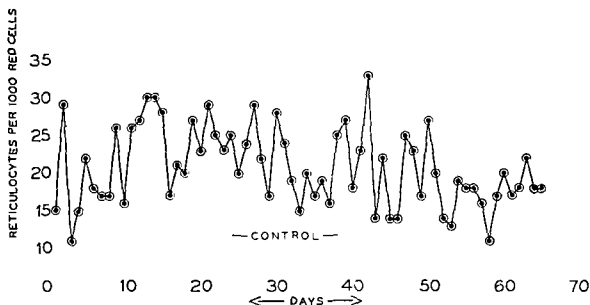


Fig. 1.—Showing the reticulocyte count of a rat over a 65-day period under our control conditions (Animal R-58, Table II).

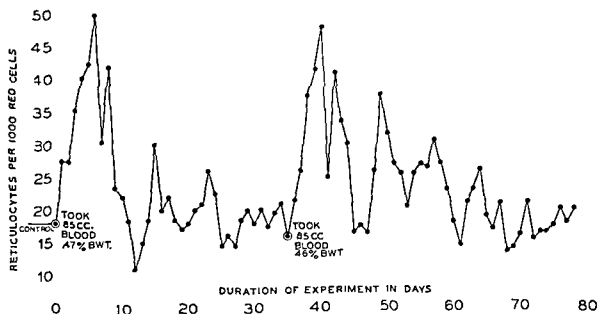


Fig. 2.—Showing the hemopoietic response to withdrawals of small but similar amounts of blood (Animal R-4).

*Stimulating Effect of Hemorrhage Upon the Hemopoietic Mechanism.*—It would seem logical to assume that hemorrhage would be a physiologic and sufficient stimulus for the prompt replacement of the blood lost. Upon this basis we proceeded to establish the relationship existing between the degree of hemorrhage and the hemopoietic response. It was found that blood losses amounting to 0.1 per cent or more of the body weight give a definite rise in the reticulocyte count within twenty-four to forty-eight hours, and when increasingly larger amounts are lost, the responses are proportionately greater. The effective stimulus, however, persists for a longer time following extensive blood loss (see Table III).

TABLE III  
SHOWING THE RELATIONSHIP BETWEEN HEMORRHAGE AND RETICULOCTYE RESPONSE IN A SERIES OF GRADUATED EXPERIMENTS

ANIMAL NUMBER	WEIGHT	SEX	CONTROL LEVEL OF RETICULOCTYES	BLOOD WITHDRAWN		LATENT PERIOD OF REACTION	PEAK REACTION	TIME REQUIRED	
				As % Body Weight	As c.c.			MAXIMUM RESPONSE	RETICULOCTYE TOSIS TO SUBSIDE
	Grams		Per 1,000 R.B.C.	Control	Control	Days	Reticuloctyes Per 1,000 R.B.C.	Days	Days
R-8	140	F	20	0.1	Control	Control	26	Control	Control
R-8	170	F	20	0.23	0.17	1	37	4	1
R-12	209	M	21	0.36	0.48	1	48	2	2
R-10	140	F	20	0.6	0.5	2	47	4	5
R-11	219	M	19	1.0	1.31	1	57	3	6
R-7	148	F	19.5	1.5	1.48	1	92	3	7
R-13	205	M	19	2.0	3.01	2	77	5	9
R-14	176	M	17	2.5	3.52	1	103	3	9
R-9	140	F	19.7		3.5	1	114	2	18

In Table III there appear four significant columns of data: (1) reticulocytes per 1,000 red blood cells; (2) latent period of reaction; (3) time required for maximum response, and (4) time required for reticulocytosis to subside.

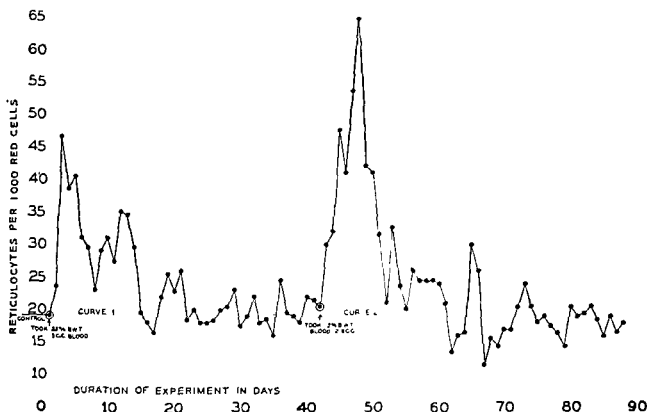


Fig. 3.—Showing hemopoietic response to withdrawals of dissimilar amounts of blood. The initial hemorrhage in this case must be less than 0.5 per cent of the body weight, or preferably must produce a submaximal response, otherwise the reaction to the second withdrawal may be distorted (Animal R-2).

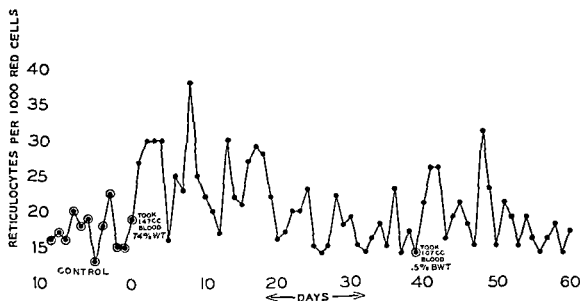


Fig. 4.—Showing that animals which react only slightly to an initial withdrawal of blood tend to retain this characteristic (Animal R-52).

(1) With regard to the reticulocyte count, it is apparent that this indicates the reaction of the bone marrow to hemorrhage, and in the ideal physiologic state should be in direct proportion to the extent of the blood loss. In our experiments we found this situation to obtain within limits. There are, however, two instances (specifically animals R-10 and R-13, Table III) where this straight line effect did not occur in our experiments. The peak reticulocytosis did not rise proportionally above that of the preceding experiment where a lesser hemorrhage was induced. It is unfortunate that in our experiments upon

rats where the animal is small and very reactive, it is not permissible to make enough blood smears to locate exactly the peak reaction. If this were possible, we might easily clear up this exerescence. In support of our contention, however, that there is a direct quantitative relationship between stimulus and response; note that a progressively longer time is required for the return of the reticulocyte count to control level, indicating that the reaction was greater than the peak count would indicate. As already stated this relationship of stimulus

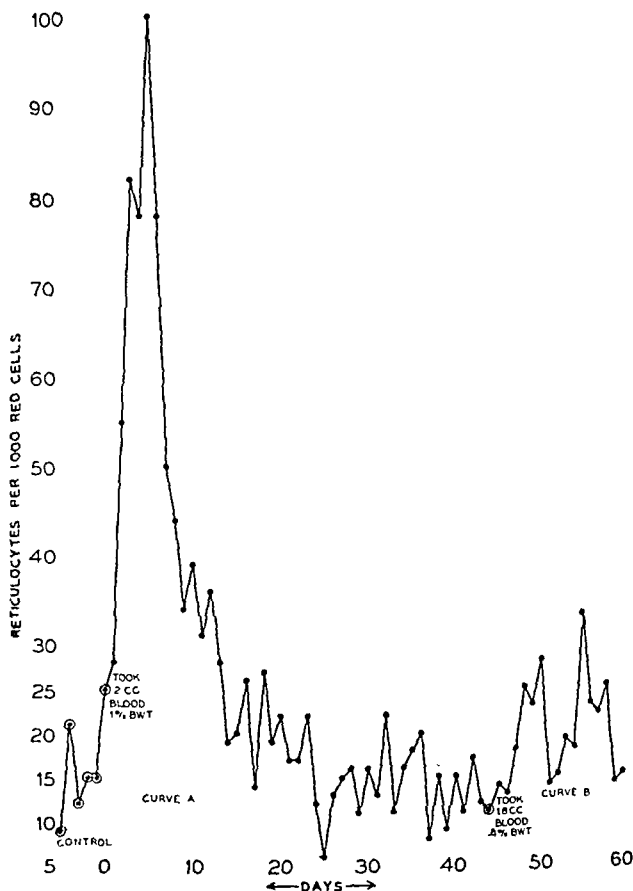


Fig. 5.—Showing that an initial maximum response of the hemopoietic system may be so exhaustive to the bone marrow that neither spontaneous reticulocyte showers nor subsequent typical response to severe hemorrhage occurs (Animal R-54).

to response applies only within limits. When the stimulus (hemorrhage) approaches maximum proportions, the response can be maximum only for the set of new conditions established, and any further depletion of the animal's blood hampers rather than facilitates hemopoiesis. Evidence in support of this is discussed under the "Effects of Repeated Hemorrhage" (see Fig. 5).

(2) *The latent period* throughout the experiment was consistently between twenty-four and forty-eight hours (Table III).

(3) *The time required to reach a maximum reaction*, whether in response to a mild or severe hemorrhage, is from two to five days, the average being approximately three days (Table III).

(4) *The time required for the reticulocytosis to subside* is in direct proportion to the degree of hemorrhage (Table III).

*Effect of Repeated Hemorrhage Upon the Hemopoietic Mechanism.*—The question of how often and to what extent the bone marrow can accomplish replacement of cells lost is pertinent. We have, therefore, repeated the withdrawal of blood upon a number of animals immediately after the reticulocyte count returned to and was maintained at control level and have been able to demonstrate that (1) loss of comparable small quantities of blood, i.e., up to 0.5 per cent of the body weight gave rise to similar responses and complete replacement (Fig. 2); (2) loss of different, yet small quantities of blood gave rise to different but proportionate responses (Fig. 3); (3) in animals where the response was originally weak (in no case has it been absent) it remains weak upon repetition (Fig. 4). Following extensive initial withdrawals of blood (1 per cent of the body weight and up) repetition of the hemorrhage resulted in indefinite or very slight responses even when the withdrawals of blood were separated by fifty or more days (Fig. 5). So exhaustive to the bone marrow is the initial response in these cases that subsequent spontaneous reticulocyte showers are absent or only slightly apparent. This fact is very important to remember for present interpretation of experiments bearing upon the life duration of the red blood cell. Only bone marrow which has given initially a submaximal response can be expected to react to a subsequent stimulus furnished either by a second withdrawal of blood or by the mass senescence of a generation of red blood cells.

TABLE IV

SHOWING THE TYPE OF RETICULOCYTE REACTION ELICITED, AND THE NUMBER OF ANIMALS GIVING EACH TYPE OF RESPONSE

SERIES	NUMBER OF ANIMALS IN SERIES	NUMBER AND PERCENTAGE OF ANIMALS			
		SHOWING PRIMARY RESPONSE ONLY	SHOWING SECONDARY RESPONSE ONLY	SHOWING COMPOSITE RESPONSE ONLY	SHOWING INDEFINITE RESPONSE
1	4	75% (3 animals)	0	25% (1 animal)	0
2	8	87.5% (7 animals)	0	12.5% (1 animal)	0
3	10	60% (6 animals)	20% (2 animals)	20% (2 animals)	0
Total	22	73% (16 animals)	9% (2 animals)	18% (4 animals)	0

*Primary and secondary hemopoiesis probably involved:* In the whole series of 22 rats in which the bone marrow was stimulated by hemorrhage, we were able to detect four types of response: (1) a primary response (Fig. 6); (2) a secondary or latent response (Fig. 7); (3) a composite response (Fig. 8); and (4) an indefinite or slight response (Fig. 4 and Fig. 5, curve B). In all cases the "latent period" of reaction was used as the index for determining whether the response was primary or secondary, and the reticulocyte count was used as the index of the extent of the response. Table IV shows the distribution of the four types of reaction.

*Primary response gives evidence of the life duration of the red cell:* It is clear that curves of the type shown in Fig. 6 illustrate a simple prompt reaction of the hemopoietic mechanism to hemorrhage, raising the reticulocyte count momentarily to 9.2 per cent. At definite intervals thereafter (nine days) second-

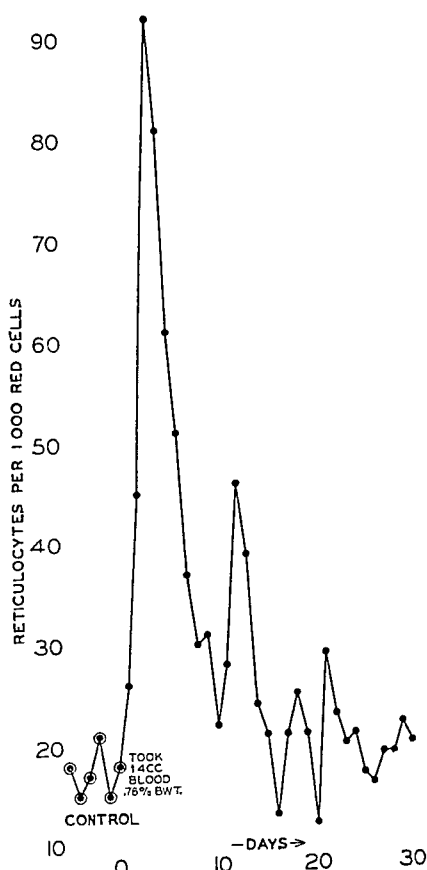


Fig. 6.—Showing simple (primary) reaction followed by two spontaneous reticulocyte showers at intervals of 9 days each (Animal R-7).

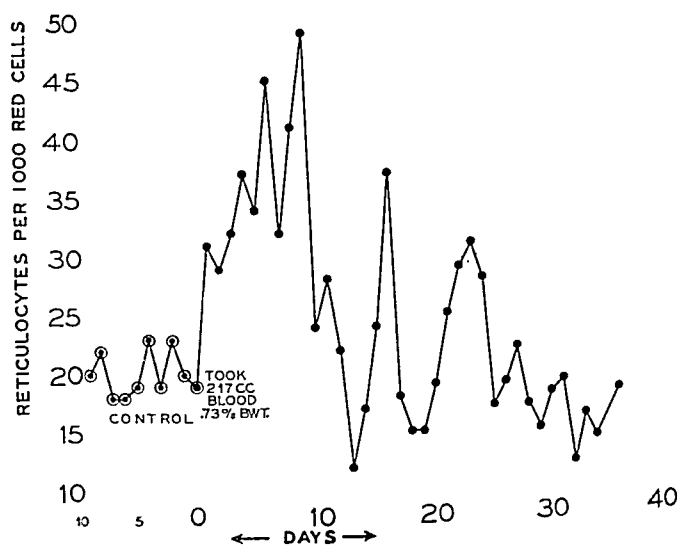


Fig. 7.—Showing the secondary type of reaction, followed by two spontaneous reticulocyte showers at 7-day intervals (Animal R-56).

Note. In Fig. 6 a corresponding but weaker response of this type is shown in addition to the main primary reaction.

ary peaks occur spontaneously. These peaks are preceded by low reticulocyte counts, showing that a subsidence of the initial reaction occurred. Subsequently a spontaneous reaction takes place resulting, we believe, from the mass senescence or destruction of all those cells which represented the initial or preceding reaction, thus furnishing the necessary stimulus. Thus being true, the life duration of the red blood cell in the rat would be represented by the time interval between reticulocyte showers which in this case is about nine days.

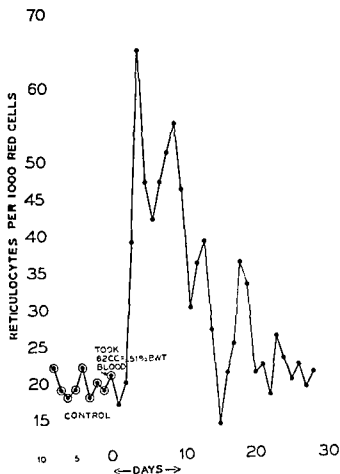


Fig. 8.—Showing the composite type of reaction. In this graph both primary and secondary reactions are seen, each followed in 9 days by its respective spontaneous reticulocyte showers (Animal R-8, series 4).

*Secondary response:* In Fig. 7 the predominant response is one requiring a nine-day period for completion. This we have interpreted as a secondary type of reaction. Again following the peak of this secondary reaction, periodic reticulocyte showers occur, but this time at seven-day intervals. This is the shortest interval found for these spontaneous showers. Whether red blood cells which have been produced by secondary hemopoiesis under stress of hemorrhage become senescent at an earlier age than those furnished by the more prompt primary hemopoiesis, our experiments do not definitely show. The two animals of our series which gave this latent type of response showed spontaneous showers at seven and eight days, respectively.

*Primary and secondary responses:* Fig. 8 is a typical example of the most complicated curve of our series. In it both primary and secondary responses occur, followed by their respective spontaneous showers. The initial primary peak occurs in four days; the secondary in nine days. By counting nine days (it varies in different rates from seven to nine days) from the peak of either the primary or the secondary reaction, one may establish responsibility for all the intermediate spontaneous reticulocyte showers.



*Indefinite types of response:* As already mentioned, not all animals show the typical primary or secondary reaction to hemorrhage under all conditions. Also a most striking deviation is that found when a second hemorrhage follows a typical reaction from a loss of blood of from 1 per cent of the body weight up (see Fig. 5, curve B). It appears that even though the bone marrow is capable of initial replacement of blood loss; it is not able to repeat the process with the same effectiveness even after protracted periods. This not only is demonstrated in our repeat experiments, but also is evident in all those experiments where marked initial responses occurred so as to prevent the appearance of any spontaneous reticulocyte showers. Therefore, indefinite responses, as we have observed them, may be found occasionally in seemingly normal animals (Fig. 4), and in reactive animals following severe hemorrhage (Fig. 5, curve B).

It is clear from this fact that to determine the life span of the red blood cell by a study of the spontaneous reticulocyte showers following hemorrhage, the stimulus must be of submaximal value for primary hemopoiesis, and preferably subminimal for secondary hemopoiesis. Otherwise compound or indefinite responses becloud the results.

#### CONCLUSIONS

1. A method for making and counting permanent preparations of rat reticulocytes is described.

2. Average control values for reticulocytes in rat blood are given for 22 animals.

3. Hemorrhage was found to be a sufficient and quantitative stimulus for the rat's hemopoietic system, within the limits described.

4. The span of life of the red blood cell in the albino rat was determined by autonomous blood replacement following hemorrhage, and averages 8 to 9 days. The spontaneous reticulocyte showers which follow hemorrhage were used as the index.

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## THE EFFECT OF ALKALOSIS ON THE BLOOD PICTURE IN CHRONIC SPLENOMYELOGENOUS LEUCEMIA\*

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SINCE Menten<sup>1</sup> reported that the blood pH was increased in many instances of human malignancy, there have been attempts to invoke a relationship, causal or otherwise, of alkalosis to cancer. McDonald<sup>2</sup> holds such a relationship to exist, and particularly in Europe, cancer has been treated by the administration of acid. In the case of transplanted animal tumors, Woglom<sup>3</sup> was unable to influence their growth rate by the administration of either acid or alkali, while Barnard<sup>4</sup> reported an apparent diminution in the growth rate of transplanted mouse sarcoma during periods of dietary alkalosis. Parfentjev, Devrient, Suntzeff, and Sokoloff<sup>5</sup> caused regression in a large percentage of transplanted rat tumors by the administration of lactic acid. As the latter substance was given as the sodium salt and as the lactates have an alkaline ash, it is difficult to decide from their studies whether the end result in this case was due to the effect of acid or of alkali.

We have had the opportunity of observing the blood pictures of two patients with chronic splenomyelogenous leucemia during the administration of large doses of sodium bicarbonate. That alkalosis was actually induced in these cases was adjudged from the clinical symptoms. Bradypnea, prostration, and generalized anasarca developed concomitantly with the administration of the alkali but subsided upon the cessation of administration. The clinical picture of alkalosis is a much better criterion for the development of this state than the determination of blood pH.†

It was thought that such observation might indicate to us whether or not there was any stimulation of neoplastic processes as a result of alkalosis. Whereas bone marrow studies would probably have been more conclusive, they were unavailable. Nevertheless, we feel that the data presented, when analyzed, indicate that there is no stimulation of neoplastic tendency, at least in this particular neoplastic disease, by the induction of alkalosis.

The patients on whom the study was made were adult males, one in the fourth and the other in the sixth decades. Both had had courses of roentgenotherapy; one, three years, and the other one year before the present study. Their conditions were clinically stationary at the time, and each consented to the alkalization procedure. Both patients died shortly after the data were obtained; one from cerebral embolism, two weeks following the last dose of alkali

\*From the Therapeutics Service of the Cook County Hospital, Chicago.

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†See in this connection the authors' editorial in the J. A. M. A., July 27, 1931.

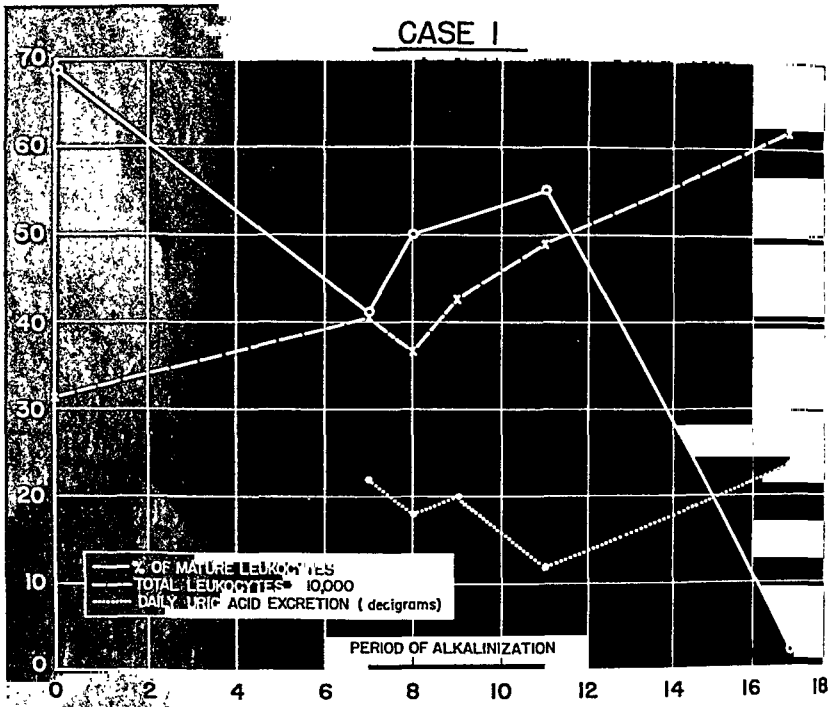


Fig. 1.

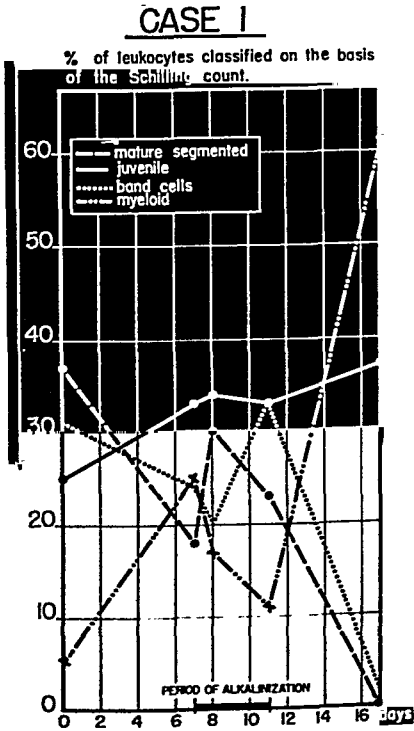


Fig. 2.

(Case 2); and the other patient, we were informed, died about a month following the last observation. Although the relationship of the alkalosis to the deaths of these patients is open to question, we have not held it justifiable to repeat the procedures.

Fig. 1 gives the essential data on Case 1. The Schilling<sup>6</sup> count during the period of alkalization shows a definite trend toward the right, though before this period it is apparent that the patient was headed for a relapse since the hemogram was shifting to the left. Upon cessation of the administration of alkali, the patient was given magnesium sulfate, 20 gm. per day, to assist in removing the edema fluid that had accumulated during the period of alkalosis. It is of interest in this connection that divalent cations like magnesium are classed as "acidifiers" when given orally (Van Slyke and Peters<sup>7</sup>).

That the increase in total cells in the circulation is due to persistence of mature forms in the blood rather than to an increased production of leucocytes, is inferred from the uric acid excretion, which was followed in Case 1 and which fell during this period.

Fig. 2 shows the distribution of cells in the hemogram of Case 1. The greatest drop during the alkalosis period was in the least mature (myeloid) cells, the *percentage* of mature cells rising most.

TABLE I

DAY	NaHCO <sub>3</sub> PER DAY	W.B.C.	% FILAMENT	IMMATURE CELLS PER C.M.M.
1	20 gm.	523,600	28.2	375,945
2	20 gm.			
3	20 gm.			
4	50 gm.	521,000	32.8	350,112
5	50 gm.			
6	50 gm.			
7	50 gm.			
8	50 gm.			
9	50 gm.			
10	50 gm.			
11	50 gm.			
12	--	567,500	39.6	342,770

Table I shows the result of alkali administration on the filament-nonfilament cell ratio in Case 2. Here again, there is a definite shift to the right during alkalosis. We did not succeed in obtaining blood smears after the period of alkalization was over. The shift, however, during the period of observation, is in the opposite direction from that to be expected were there any stimulation of myeloblastic activity.

#### SUMMARY AND CONCLUSIONS

The induction of a period of alkalosis by the administration of large quantities of sodium bicarbonate to two patients with splenomyelogenous leucemia, did not stimulate myeloblastic activity as determined from a study of their hemograms. In fact, there seemed to be a tendency toward inhibition of leucopoiesis during this period. Since myeloblastic activity, in this condition, is presumably neoplastic, it is concluded that this study has furnished evidence against the relationship of alkalosis to neoplasia.

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## OBSERVATIONS ON REDUCING SUBSTANCES IN THE BLOOD PLASMA OF RHEUMATISANTS\*

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### INTRODUCTION

A CONSIDERABLE series of observations has been conducted by one of us (R. P.)<sup>1</sup> and his associates upon the metabolic deviations encountered among patients with chronic rheumatoid diseases. Early in the course of these studies it was noted that certain persons with arthritis presented evidence of a delayed rate of removal of glucose from the blood following ingestion of this carbohydrate. In a number of such patients convalescence, when associated with significant therapy such as the removal of foci of infection, was marked by a return to a normal "glucose tolerance." The experimental evidence available to date has suggested strongly that the delayed rates of removal of glucose in question represent chiefly or wholly a disturbance in the circulation, especially in the finer vascular channels. This view is supported by the fact that the respiratory quotient of such patients is normal.<sup>2,3</sup> There has thus been little basis for assuming a contributory deviation of pancreatic function. Such a possible deviation has not been eliminated, however, and the appearance of a new method for fractionating reducing substances in the blood suggested the possibility that further data bearing upon this question might be obtained. The method, described by Polonovski,<sup>4</sup> for estimation of chromic acid equivalents provides a means of partitioning the reducing substances. It appeared that the application of this procedure to groups of rheumatic subjects would show whether an excess of intermediary products of carbohydrate metabolism accumulate in the fluid tissue of arthritic persons. Another point of incidental interest in evaluating these fractions is derived from the fact that the nonglucose fraction includes pyruvic acid, a substance known to be increased in states of

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vitamin B<sub>1</sub> deficiency. This consideration is relevant in that certain clinical symptoms of chronic arthritis are regarded as possibly due to a state of inadequacy with respect to this vitamin. For these reasons, observations have been conducted upon a series of 55 patients with chronic arthritis with respect to the levels of reducing substances in the fasting blood plasma. Further observations have been made upon the levels of the reducing substances in the blood plasma of 17 subjects in the same series following the administration of glucose by mouth.

#### PROCEDURE

The procedure for estimating the sulfuric-chromic acid indices of reducing substances has been extensively used in France. Since a description of the method may not be generally available, an explanatory account of the slightly modified procedure employed in the present studies is presented. Venous blood from fasting subjects is transferred to a test tube containing about 100 mg. of sodium fluoride. The sodium fluoride prevents coagulation and inhibits glycolysis. The specimen is centrifuged for ten to fifteen minutes at 3,000 r.p.m. to separate the cellular elements. All glassware used in the procedure is kept in sulfuric-chromic acid cleaning solution and thoroughly rinsed with water just before use. Two milliliters of plasma are added to 2 ml. of sodium tungstate solution (10 per cent) contained in a 15 ml. centrifuge tube. After mixing with a glass rod, 2 ml. of a solution of sulfuric acid (2/3 N) are added and mixed. After standing fifteen to twenty minutes, the mixture is diluted with 4 ml. of distilled water and again mixed. After centrifugation 2 ml. of the supernatant fluid is transferred to a test tube containing 10 ml. of sulfuric-chromic acid mixture (N/10). (This mixture is prepared by combining 250 ml. of a solution, containing 1.96 gm. K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> per 100 ml. of water, with 250 ml. of concentrated sulfuric acid in a 1,000 ml. flask, and diluting to volume with water.) A blank determination is run with 2 ml. of distilled water replacing plasma. The mixture is heated in a boiling water bath for one hour. The mixtures are stirred at ten-minute intervals with a glass rod. After heating, the tubes are cooled in running water, and the contents, together with the water used in washing, transferred to Erlenmeyer flasks, making a final volume of approximately 75 ml. One milliliter of potassium iodide solution (50 per cent) is added, mixed gently, and sodium thiosulfate (N/10) is run in from a burette until the solution presents a pale yellow color. At this point 4 drops of starch solution (1 per cent) are added, and the titration is continued to the usual end point. The difference between the volume of sodium thiosulfate required for the blank and the amount required to reach an end point with the deproteinized plasma represents the amount of chromic acid reacting with the reducing substances in 0.4 ml. of original plasma. This quantity, when multiplied by 2.5, shows the amount of N/10 chromic acid used by 1 ml. of plasma, and is designated as the I.C.T., or index chromic total. According to the usual terminology, this represents the total reducing substances in terms of chromic acid equivalents. A glucose determination is made at the same time with 2 ml. of the protein-free supernatant fluid, using the Benedict<sup>5</sup> method. The value so obtained, when multiplied by 1.33 (factor for conversion to the chromic acid equivalent for glucose) is called the I.C.G., or index chromic glucose. This value might be known simply as the

glucose reducing equivalent. The difference between the total and the glucose fraction is called the I.C.R., or index chromic residue. This, according to the conventional usage, may be called the nonglucose reducing fraction in terms of chromic acid equivalents.

## CALCULATIONS

I. C. T. = Titration of blank milliliters—titration of plasma milliliters  $\times \frac{5}{2} \times f$

$f$  = factor for  $\text{Na}_2\text{S}_2\text{O}_3$

I. C. G. = Glucose (mg./ml.)  $\times 1.33$

I. C. R. = I. C. T. - I. C. G.

TABLE I

LEVELS OF REDUCING SUBSTANCES IN THE BLOOD PLASMA CLASSIFIED ACCORDING TO CLINICAL GROUPS

TYPE	CASES	DETERMINATIONS	REDUCING FRACTIONS CHROMIC ACID EQUIVALENTS			
			RANGE	TOTAL I.C.T.	GLUCOSE I.C.G.	NONGLUCOSE I.C.R.
Atrophic	23	27	Max.	2.28	1.53	1.04
			Min.	1.09	0.93	0.09
			Avg.	1.53	1.10	0.43
Hypertrophic	14	16	Max.	2.40	1.97	0.78
			Min.	1.30	0.93	0.24
			Avg.	1.69	1.20	0.49
Mixed	11	14	Max.	2.18	1.38	0.95
			Min.	1.33	0.88	0.21
			Avg.	1.72	1.10	0.61
Fibrositis	3	3	Max.	1.56	1.19	0.60
			Min.	1.39	0.96	0.31
			Avg.	1.48	1.06	0.42
Miscellaneous rheumatoid*	4	9	Max.	2.16	1.50	0.66
			Min.	1.5	1.06	0.44
			Avg.	1.73	1.20	0.53
Nonrheumatoid controls	2	2	Max.	1.64	1.20	0.44
			Min.	1.36	1.06	0.30
			Avg.	1.50	1.13	0.37

\*Dupuytren's contracture, atypical atrophic, pituitary obesity, possible gonococcal arthritis.

## DISCUSSION

The patients included in this series are often classified as arthritic or rheumatic. However, both of these terms hold special implications: the former, articular inflammation; the latter, rheumatic fever. We suggest, therefore, the term *rheumatisants* (Dorland's Medical Dictionary) as descriptive of the series. This is an English word derived from the French, having in current usage no limiting concepts, and hence suitable to designate persons with rheumatism or arthritis in the general sense. The subjects are classified according to the usual balanced clinical evaluation, and are designated as atrophic, hypertrophic, mixed arthritis, fibrositis, and miscellaneous rheumatoid groups, the latter including those persons who cannot be classified under any other group. The data arranged according to such a classification are presented in Table I. The maximal and minimal values for each fraction of reducing substance, namely, I.C.T., I.C.G.,

and I.C.R., are presented for each class. In addition, the arithmetical average for each class is shown. The number of nonrheumatoid controls presented here is shown merely to indicate the general correspondence of these data with other series. The extensive data of Warembourg<sup>6</sup> show that normal I.C.R. levels of reducing substances vary from 0.30 to 0.50, and the present figures for normals fall within these limits. No attempt has been made to repeat the work of others as to the varieties of disease in which comparable deviations occur. Levels higher than normal have been found by several observers in diabetes, terminal stages of nephritis, and certain hepatic diseases. It is obvious that deviations from normal are not pathognomonic for any particular clinical state. The range of values encountered in each group of rheumatisants, however, is rather wide and tends to be outside that encountered among normal subjects. Inspection reveals that the average I.C.G. fractions (reflecting the amount of glucose) vary among the several groups from 1.06 to 1.20, thus differing by a maximum of only 13.7 per cent. The average I.C.R. fractions (reflecting the amount of nonglucose reducing substances) among the several groups vary from 0.42 to 0.61, thus differing by an average of 45 per cent.

Patients classified as atrophic or fibrositic show average I.C.R. levels similar to those of normal persons. Such averages, however, tend to obscure certain facts which are evident in the tabulations of individual cases. Thus certain individual members of the atrophic class show abnormally low figures. Some individual members of the hypertrophic group also show abnormally low figures. It is not possible at present to correlate these figures with the clinical features which characterize these patients.

On the other hand, the hypertrophic, and particularly the mixed, arthritic persons show, when averaged, group levels of nonglucose reducing substances in excess of normal. The data, as a whole, suggest that the conventional clinical groupings of arthritic persons, based upon the microscopic and gross morbid anatomy of the joints, do not fully parallel the physiologic deviations indicated. In this connection note may be made of the few extremely low values for I.C.R. encountered in certain subjects. Subnormal levels may be, conceivably, attributed to a relative deficiency of vitamin C. It may be observed further that those persons presenting levels of nonglucose reducing substances higher than normal may largely reflect an increase of pyruvic acid and hence may be regarded as subjects of possible deficiency of vitamin B. The data presented are not sufficiently clear-cut on these points to provide a basis for generalization. Therapeutic feeding experiments with vitamin C in the former instance, and with vitamin B in the latter, are necessary in order to provide a crucial test of these possibilities.

The afore-mentioned studies were conducted upon fasting subjects. In order to clarify some of the questions arising therefrom, studies were also made upon the rate of removal of glucose, following alimentary administration, on 17 patients included in this series. Such observations have been made upon 7 atrophic arthritic persons, 3 hypertrophic arthritic persons, 6 mixed arthritic persons, 1 rheumatoid person with pituitary disturbances, and 3 normal persons. The glucose curves in this series are, for the most part, within the normal limits of variation. However, a few present evidence of a slightly delayed rate of glucose re-



TABLE 11

LEVELS OF REDUCING SUBSTANCES IN BLOOD PLASMA FOLLOWING INGESTION OF GLUCOSE

PATIENT	MEAL	TIME	GLUCOSE MG. PER 100 ML.	REDUCING FRACTIONS CHROMIC ACID EQUIVALENTS			CLINICAL NOTES
				TOTAL I.C.T.	GLUCOSE I.C.G.	NON- GLUCOSE I.C.R.	
B.	Glucose with lemon juice	Fasting	75	1.36	0.99	0.37	Atrophic, without evident foci of in- fection
		1 hour	110	2.16	1.46	0.70	
		2 hours	90	2.06	1.19	0.87	
		3 hours	80	1.52	1.06	0.46	
M.	Glucose alone	Fasting	92	1.57	1.22	0.35	Atrophic, with pro- statitis
		1 hour	128	3.30	3.03	0.27	
		2 hours	170	2.72	2.26	0.46	
		3 hours	122	2.09	1.62	0.47	
P.	Glucose with lemon juice	Fasting	92	1.85	1.22	0.63	Hypertrophic, with- out evident foci of infection
		1 hour	205	3.50	2.72	0.78	
		2 hours	170	3.24	2.26	0.98	
		3 hours	130	2.50	1.72	0.78	

removal. Such delayed rates are most apparent in the severely ill active atrophic arthritic persons who might, according to some students, be classified as "infectious" arthritic persons; and in hypertrophic arthritic persons, in whom the greater average age of the group may contribute toward a "decreased glucose tolerance."

Many of the patients in this series had previously been treated for removal of foci of infection and were not, at the time of observation, under the influence of such infective factors. The comparatively low incidence of delayed rates of glucose removal in contrast to the higher incidence of abnormal curves in earlier observations may be due to this fact. However, the patients under observation presented clinical evidence of disability and a certain degree of arthritic activity. The data in 3 representative cases are presented in detail in Table II to illustrate the salient features encountered. A few of these observations were conducted with a routine hospital procedure, which includes the administration of lemon juice as a component of the glucose solution. Lemon juice contains reducing substances other than glucose. Only those subjects who received lemon juice with the glucose showed significant increments in the nonglucose fractions. This suggests that there is no primary disturbance in carbohydrate metabolism in the instances which show a high curve.

## SUMMARY

Observations have been made upon a series of 55 patients with chronic arthritis of varied types in respect to the levels of substances having the common property of reducing chromic acid. These consist of glucose and of nonglucose materials. The blood plasma of rheumatisants presents deviations from normal mostly in the direction of an excess of reducing substances other than glucose. These deviations from normal are not confined to any one group of rheumatic subjects. Within the clinical pathologic groups discussed, wide individual variations are noted, the physiological significance of which is not clear. The distribution of reducing materials in the blood following alimentary administration sup-

ports the view previously suggested in principle that, when the rate of removal is delayed, the disturbance is not one of specific interference with the metabolism of glucose, but is probably referable to a circulatory mechanism.

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## BLOOD CYTOLOGY OF THE NORMAL DOG\*

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SINCE the dog is commonly used as an experimental animal, numerous studies involving the hematopoietic system have appeared in the literature. Although a large majority of these data are comparable, the extreme variation indicates that all the information was not obtained upon dogs which were in a similar state of health, nor were the dogs examined under the same conditions. An excellent survey of this type of literature on the blood picture of normal dogs was made by Scarborough<sup>1</sup> in 1930. It is possible to determine, with a fair degree of accuracy, the blood cytology of normal adult dogs from this survey. The same year Mayerson<sup>2</sup> reported on the blood cytology of 60 normal dogs, and his results are in approximate agreement with those summarized by Scarborough.

It is questionable whether data collected under the conditions of experimental laboratories should be used to establish normal indices for dogs examined under the routine of a small animal hospital. Experimental dogs are usually of mixed breeds taken from the pound, and their previous history is unknown. Many dogs received at hospitals are refined breeds with known histories. Furthermore, the literature does not contain sufficient information concerning the blood picture of young dogs, and a large percentage of hospitalized animals are puppies.

The present investigation was undertaken, therefore, to establish average indices for normal dogs of various breeds and ages which were received and ex-

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amined under routine hospital conditions. The examination included the owner's history of the dog, clinical observations by a veterinarian specializing in small animal practice, examination of the feces for parasites, and blood and urine analyses if they were requested by the clinician. Sixty-six normal dogs were selected from approximately 1,000 animals which represent a cross section of the clinic. Most of the young dogs used in this study were brought to the hospital for distemper prophylaxis and were free, or practically free, of intestinal parasites and were in excellent health. The older dogs were not always entirely free from intestinal parasites and some of them had minor disorders, but according to our experience, they were in better condition than the average normal dog selected from a pound for such studies. The data should give indices for blood cytology that can be used as a future reference for interpreting the results of the laboratory on abnormal animals.

#### METHODS

Blood was obtained from the radial or saphenous veins. A 20 gauge needle was used on all dogs except the very small ones, where a 25 gauge needle was used. The vein was distended by pressure and the needle immediately inserted. While the blood was flowing freely from the needle, samples for the erythrocyte and leucocyte counts were collected directly in Thoma pipettes according to the standard technique. Hayem's solution was used to dilute the blood for the erythrocyte count, and 2 per cent acetic acid was used as a diluent for the leucocyte count. A 20 c. mm. sample was diluted 1:400 with N/10 hydrochloric acid for the hemoglobin determination. The needle was then removed from the vein, and small drops were forced from the point onto clean glass slides. A heavy cover glass, such as is used for a counting chamber, was then touched to the blood, and thin even smears were prepared by drawing the blood along the slide.

The pipettes were enclosed in rubber bands to prevent losses and evaporation. The samples were shaken by hand for two minutes and the first 2 to 3 drops were blown from the pipette. A Hellige counting chamber, with improved Neubauer rulings, was then filled. The leucocytes were counted immediately while the erythrocytes were allowed to settle for about two minutes. The four corners (64 large squares) were counted for the leucocytes and 80 small squares for the erythrocytes. When distribution was quite even, only one chamber was counted.

Blood smears were stained with Wright's stain. The differential leucocyte count was made by counting 100 cells according to the meander method as outlined by Gradwohl.<sup>3</sup> The cells were classified into various groups, using the Schilling method of classification. With this method, the polymorphonuclear neutrophils are classified as, myelocyte, juvenile, stab, or segment. The first two forms are very rarely found in the blood of the normal dog. In order to classify a cell as a stab, the nucleus had to have the typical band form. The nucleus of the segments of the dog is not well divided, or segmented, as are those of man. If the neutrophils of the dog were classified by Armeth's method, there would apparently be a shift to the left in comparison with the values found normal for man.

The work reported here was done by eight trained technicians. However, 60 of the counts were about equally divided among four of them. The error in

the enumeration of the cellular elements was between 5 and 10 per cent. According to controlled tests, the error for the erythrocyte counts was about 300,000, and for the leucocyte counts about 500.

Hemoglobin determinations by the Newcomber method were accurate to within 3 per cent. The determinations made on the Haden-Hausser hemoglobinometer were to the nearest 0.5 gm.

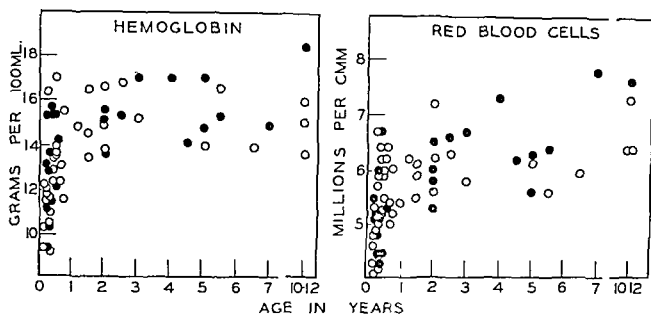


Fig. 1.—Grams of hemoglobin per 100 ml of blood and millions of red blood cells per cubic millimeter of blood in 66 normal dogs are plotted against age. The open circles represent data from males; the closed circles data from females.

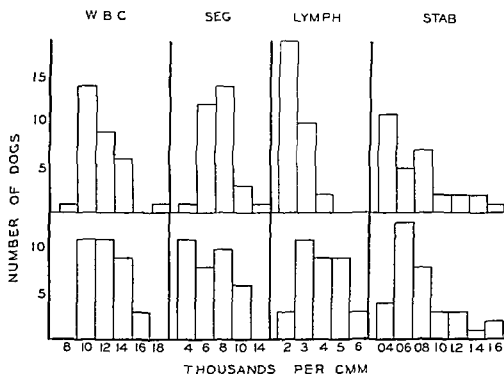


Fig. 2.—The distribution of white blood cells, segments, lymphocytes, and stabs is shown for 35 dogs, 2 to 8 months old, in the lower series of plots, while similar distributions are illustrated for 31 dogs, 9 months to 12 years old, in the upper plots.

## RESULTS

The data in Table I and Fig. 1 demonstrate that the number of red blood cells and the concentration of hemoglobin increase from 2 months of age to puberty, which is approximately 8 months in a majority of the breeds investigated. The average red cell count for the 35 dogs from 2 to 8 months, inclusive,

TABLE I  
DATA OBTAINED ON 66 NORMAL DOGS OF VARIOUS AGES AND BREEDS

DOG NO.	BREED	AGE	SEX	HB. GMS. PER 100 G.G.	R.B.C. MILLIONS PER C. MM.	W.B.C. THOUSANDS PER C. MM.	SEG.	LYMPH.	STAB	EOSIN.	MONO.
43	Scottish terrier	2 mo.	M	10.2	4.3	12.0	7,320	3,120	1,560	---	---
65	Mongrel	2 mo.	M	9.3	4.6	10.3	3,811	5,150	1,030	309	---
484	German shepherd	2.5 mo.	F	15.3	5.5	14.1	5,640	5,500	987	1,974	---
1,000	Cocker spaniel	2.5 mo.	M	12.1	4.8	9.5	6,550	2,980	570	95	---
1,025	English setter	3 mo.	M	16.3	5.3	12.5	4,875	6,875	750	---	---
1,023	Collie	3 mo.	M	12.0	4.9	12.1	4,598	5,929	726	847	---
1,026	Springer spaniel	3 mo.	M	11.4	4.1	9.2	4,324	3,772	552	552	---
1,024	Collie	3 mo.	F	13.1	5.1	12.7	6,350	4,372	1,270	508	---
1,027	Cocker spaniel	3.5 mo.	F	11.1	5.2	11.5	5,865	4,370	575	690	---
437	Cocker spaniel	3.5 mo.	M	11.7	5.7	13.6	8,840	2,992	680	1,088	---
116	Labrador retriever	4 mo.	F	9.3	4.8	11.9	8,092	3,213	595	---	---
457	Cocker spaniel	4 mo.	M	12.8	6.2	10.0	6,000	3,500	500	---	---
665	Cocker spaniel	4 mo.	F	15.3	4.5	9.0	4,500	2,700	1,800	---	---
1,003	Chow Chow	4 mo.	F	12.8	6.0	13.6	9,792	2,992	680	136	276
1,004	Smooth-haired terrier	4 mo.	M	11.6	6.7	13.8	7,866	4,992	828	138	---
1,030	Bengle	4 mo.	M	10.9	5.0	14.5	8,700	5,075	725	---	---
1,005	Irish terrier	4 mo.	M	10.4	4.2	12.4	6,696	4,960	620	124	---
874	Boston terrier	4 mo.	F	10.2	5.1	12.9	9,159	2,967	645	129	---
109	Scottish terrier	4.5 mo.	M	9.1	4.4	14.5	9,560	3,625	580	435	---
1,028	Great Dane	4.5 mo.	M	12.8	6.4	11.0	4,840	4,950	330	880	---
449	Cocker spaniel	5 mo.	M	12.3	5.2	16.0	8,800	5,760	1,120	320	---
134	Irish setter	5 mo.	F	11.4	4.5	10.6	3,922	4,452	742	1,484	---
864	Samoyede	5 mo.	M	13.3	5.9	14.6	7,884	5,110	1,022	438	146
1,066	Dachshund	5 mo.	F	15.6	5.2	10.9	6,649	3,488	763	---	---
1,007	Cocker spaniel	5 mo.	F	15.3	6.7	10.0	4,900	4,000	400	700	---
1,008	Irish terrier	5 mo.	F	13.6	4.3	13.3	7,714	3,325	532	1,729	---
26	Dachshund	6 mo.	M	13.3	6.2	16.8	9,744	4,332	1,512	1,512	---
28	German shepherd	6 mo.	M	13.9	5.9	9.9	3,465	4,950	792	693	---
469	Welsh terrier	6 mo.	M	13.4	5.3	12.8	6,912	4,352	806	384	256
886	Scottish terrier	6 mo.	M	17.0	6.0	10.0	4,360	4,000	1,500	---	---



is 5.3 millions per c. mm., and the average hemoglobin concentration is 12.6 gm. per 100 ml. of blood. The average values for the 31 older dogs are 6.2 millions red blood cells per c. mm., and 15.1 gm. of hemoglobin per 100 ml. of blood, with mean deviations of 0.54 millions cells and 1.09 gm. of hemoglobin, respectively. The average corpuscular hemoglobin, calculated by dividing the concentration of hemoglobin in 100 ml. of blood by the number of cells is  $24 \times 10^{-12}$  gm. for the 35 young dogs, and  $24.3 \times 10^{-12}$  gm. for the 31 adult animals with mean deviations of 0.294 and 0.184, respectively.

TABLE II  
SUMMARY OF DATA ON LEUCOCYTES FROM TABLE I DIVIDED INTO TWO AGE GROUPS

	AVERAGE NUMBER	MEAN DEVIATION OF NUMBERS	AVERAGE PER CENT	MEAN DEVIATION OF PER CENT
2 months to 8 months				
W.B.C.	12,165	1,713	-	-
Seg.	6,795	1,760	55.85	9.82
Lymph.	4,051	912	33.30	8.24
Stab.	816	276	6.70	2.17
Eosm.	484	437	3.97	3.51
Mono.	19	-	0.15	-
9 months to 12 years				
W.B.C.	11,467	1,489	-	-
Seg.	7,525	1,280	65.27	6.55
Lymph.	2,491	563	21.72	4.98
Stab.	753	338	6.56	2.93
Eosin.	623	403	5.43	3.30
Mono.	77	-	0.67	-

The total white blood cell counts and the differentials for each animal are recorded in Table I, summarized in Table II, and illustrated graphically in Fig. 2. The data in Table II and in Fig. 2 have been separated into two age groups. The lower plots in Fig. 2 represent the distribution of white blood cells in dogs 2 to 8 months of age, while the upper series of plots represent the distribution in adult animals. The average total white count for the younger dogs is 12,165, which is slightly higher than the average value of 11,467 found for the adults. The average differential count for dogs 2 to 8 months, inclusive, expressed in percentages, is 62.5 per cent polymorphonuclear neutrophils, 33.3 per cent lymphocytes, 3.97 per cent eosinophiles, and less than 1 per cent mononuclear cells. The differential for the older dogs is 71.8 per cent polymorphonuclear neutrophils, 21.7 per cent lymphocytes, 5.4 per cent eosinophiles, and less than 1 per cent mononuclear cells. Mean deviations are used in Table II instead of the customary record of maximum and minimum number of cells found, because we believe that the former is a better basis for establishing the normal and for testing the accuracy of the data.

#### DISCUSSION

The average number of red blood cells in young dogs is distinctly below the average number for the adult dog. This result is in agreement with the records in the literature. Wells and Sutton,<sup>4</sup> for example, found the red blood cell count for 8 dogs from 3 to 16 days old to vary from 3.3 to 5.2 millions per c. mm. The average count was 4.3 millions. In 6 adult dogs, 3 males and 3 females, these authors found an average of 6.71 millions red blood cells, with extremes of 7.6 to 5.7. The average value for adult dogs of 6.2 millions red blood cells per c. mm.

obtained from our data is in excellent agreement with an average red blood cell count of 6.3 millions per c. mm. calculated from data on 566 dogs by 71 investigators reviewed by Scarborough. Wintrobe, Shumacker, and Schmidt<sup>2</sup> reported a higher average of 7.02 millions per c.mm. in 54 dogs. These authors found an average count of 7.16 millions in 22 male and 6.78 millions per c.mm. in 10 female dogs. Our figure compares favorably with the average of 6.49 millions red blood cells calculated from Mayerson's<sup>2</sup> data from 37 male and 23 female dogs. The average for the males in his data is 6.46, and for the females is 6.53 millions red blood cells. For all practical purposes the data presented here, as well as those appearing elsewhere, can be interpreted to mean that in normal dogs after puberty the number of red blood cells per cubic millimeter of blood is little influenced by age, sex, or breed.

As the number of red blood cells increase with age, the concentration of hemoglobin in the blood also increases. This is to be expected since our data prove that the corpuscular hemoglobin is essentially independent of age. The higher mean deviation in the corpuscular hemoglobin in young dogs, as compared to the older dogs, may be significant, but the difference between the average values of  $24.0 \times 10^{-12}$  and  $24.3 \times 10^{-12}$ , respectively, is small. These averages agree with the majority of those calculated from data on adult dogs in the literature. For example, an average of 15.6 gm of hemoglobin per 100 ml. of blood was obtained from an analysis of data reviewed by Scarborough on 120 dogs. If 15.6 is divided by the average number of cells calculated from data in his review, the corpuscular hemoglobin equals  $24.7 \times 10^{-12}$  gm. Mayerson<sup>2</sup> reported corpuscular hemoglobin equal to  $20 \times 10^{-12}$  gm. for his data on 60 normal dogs. This value is low due to an average hemoglobin concentration of 13.01 gm. and, as he suggested, may be the result of the semitropical environment of his dogs. Wintrobe, Shumacker, and Schmidt<sup>2</sup> calculated the mean corpuscular hemoglobin for 54 dogs to be  $21.2 \times 10^{-12}$  gm which is lower than our value, primarily because of the greater number of red blood cells. Our data, together with an analysis of the data in the literature, lead to the conclusion that there is no marked effect of age, sex, or breed upon corpuscular hemoglobin, or upon the hemoglobin concentration of the blood in adult animals.

The tendency for young dogs to have a higher white count, a greater proportion of lymphocytes, and fewer polymorphonuclear neutrophils than adult dogs, which is apparent in our data, has been mentioned in Scarborough's review. Our data show that the lower percentage of polymorphonuclear neutrophils in young dogs than in adult dogs is due to a lower relative number of segments because the percentage of stabs is similar in the two age groups. The mean deviation for the percentage of segments and lymphocytes is greater in the dogs from 2 to 8 months than in the adult animals. This difference would be expected since there must be an adjustment of the number of these cells to the adult condition. Our data indicate that there is no marked change in the relative number of the other cells with age. The differential count which Scarborough reported for adult dogs of 69 per cent polymorphonuclear neutrophils, 20 per cent lymphocytes, 5 per cent eosinophils, and 6.7 per cent large mononuclear and transitional forms is similar to our averages. The slightly higher percentage of polymorphonuclear neutrophils in our data may be due in part to the in-



elusion of transitional forms as stabs. The percentage of mononuclear cells in our data is lower, however, than the 1 to 5 per cent often reported.

It is evident that wherever comparisons can be made the data obtained under the routine conditions of a small animal hospital are in good agreement with the data reported from experimental laboratories. Apparently there is no essential difference between studies made on finer breeds of dogs kept as pets and the average normal pound dog. The data we have presented, therefore, will be helpful in establishing indices for blood cytology of normal young and adult dogs and can be used as a reference for interpreting abnormal and pathologic findings from the hospital.

#### SUMMARY

A study of the blood cytology of 66 normal dogs of different ages, breeds, and sexes was undertaken to establish normal indices for dogs received and examined under routine hospital conditions. The data obtained demonstrate that the red blood cells increase in number from less than 5 million per c. mm. of blood at 2 months of age to an average of 6.2 millions in the adult. The corpuscular hemoglobin is essentially constant for all ages and equal to approximately  $24 \times 10^{-12}$  gm. The hemoglobin concentration in the blood, therefore, also increases from 9 to 10 gm. per 100 ml. of blood at 2 months of age to an average of 15.4 gm. in the adult animals. There is no marked effect of age, breed, or sex upon the corpuscular hemoglobin in normal young or adult dogs nor on the concentration of hemoglobin in the blood of the adult dog.

The average white blood cell count in 35 dogs from 2 to 8 months of age, inclusive, is 12,165 per c. mm., which is slightly higher than the average count of 11,467 for 31 adult animals. The average differential count for the young dogs is 55.8 per cent segments, 33.3 per cent lymphocytes, 6.7 per cent stabs, 3.97 per cent eosinophiles, and less than 1 per cent mononuclear cells. The differential count for adult dogs is 65.27 per cent segments, 21.7 per cent lymphocytes, 6.56 per cent stabs, 5.43 per cent eosinophiles, and less than 1 per cent mononuclear cells. The lower percentage of polymorphonuclear neutrophils in the young dogs is due to a smaller relative number of segments, the stabs remaining constant.

These results will be used in the future as a reference for interpreting abnormal and pathologic findings.

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# SPUTUM STUDIES IN PNEUMONIA: THE EFFECT OF SULFANILAMIDE\*

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RECENTLY Price and Myers<sup>1</sup> have attempted to evaluate sulfanilamide as a therapeutic agent in the treatment of pneumococcic pneumonia. In conjunction with the above investigation, 70 roentgenographically proved cases of pneumonia† were studied, and the effect of sulfanilamide therapy upon the pneumococci in the sputum was observed. The type distribution, the incidence of bacteriemia, and the outcome are listed in Table I. For details of dosage and the clinical analysis of results, the reader is referred to the paper by Price and Myers.

TABLE I

TYPE	CASES	BACTERIEMIA	DEATHS
I	18	5	1
II	22	10	3
III	6	3	4
IV	3	1	0
V	1	1	0
VII	7	0	1
VIII	3	2	1
Others	10	4	1
Total	70	26—37 per cent	11—16 per cent

Only those patients who produced either bloody or rusty sputum have been included in the above series. Thin smears were made directly from rusty portions of the sputum and treated with Wright's blood stain. Specimens were obtained before therapy was instituted and, when possible, at twelve-hour intervals throughout the acute stages of the disease. The number of pneumococci per oil immersion field and the degree of phagocytosis were determined in the manner previously described.<sup>2, 3</sup>

## RESULTS

The effect of serum therapy upon the pneumococci in the sputum has already been reported<sup>2, 3</sup> and in brief may be summarized as follows: In samples of sputum obtained before serum treatment was instituted, the organisms usually appeared as individual encapsulated diplococci which were evenly dispersed throughout the slide. Relatively few were being phagocytized (Fig. 1). The intravenous injection of specific horse or rabbit antibody was followed by the appearance of clumps of encapsulated pneumococci in the sputum, and in some cases by a definite increase in phagocytosis. The number of extracellular en-

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†The patients received serum either before or coincidental with sulfanilamide therapy.

encapsulated pneumococci per oil immersion field gradually decreased so that by the time the sputum lost its rusty character, only a few were present. Occasionally specimens of sputum from patients who had not yet received therapy contained encapsulated pneumococci which were already clumped. In these cases it was felt that the spontaneous clumping was indicative of a developing active immunity.<sup>3</sup>

The major effect of sulfanilamide, as seen in the sputum, was entirely different from that observed when serum was the therapeutic agent. Approximately twelve hours after the drug was administered, the number of extracellular encapsulated diplococci per oil immersion field began to decrease. Within twenty-four to thirty-six hours it was frequently difficult to find pneumococci in samples of rusty sputum even though the pretherapy specimen had contained many. The remaining organisms were evenly dispersed and showed no evidence of clumping. Some minor changes, however, were noted in the pneumococci themselves. They frequently varied in size and shape. Chaining was common, and often one pair of cocci in a chain stained deeply, while others were scarcely visible. No distinct alteration in the size of the capsule and no increase in phagocytosis attributable to sulfanilamide have as yet been detected.

The effect of sulfanilamide in contrast to that of serum is presented in Table II. The cases cited have, for the most part, been selected because the sputa contained fairly large numbers of pneumococci before therapy was instituted. The effect of the drug in these patients was more striking than in those with only a few organisms per field. However, similar results were obtained in practically all of the recovered cases. Since the data from those patients who died are too meager, as a whole, to warrant discussion at the present time, only one such case is presented. The remaining patients listed in Table II recovered. The figures given represent the number of extracellular encapsulated pneumococci per oil immersion field before therapy was instituted, compared with the average count of specimens examined each day until the patients no longer produced rusty or bloody sputum.

One may note from Table II that in the serum-treated cases the appearance of clumps of extracellular pneumococci was directly related to therapy. After clumping had occurred, the number of pneumococci per field gradually decreased. Similar observations have been made in 65 additional patients treated with serum. In exceptional cases, a considerable increase in pneumococci was noted even in the presence of clumping.

Most of the patients treated with sulfanilamide showed a prompt drop in number of free pneumococci per field within twenty-four to thirty-six hours after therapy was instituted. This effect was maintained throughout the period during which rusty sputum was obtained and was not related to the presence of clumping (see Case, D.F., F.D., E.E., and D.C. in Table II). In a few of those patients who showed the initial decrease, the effect was not maintained. Within two to four days, in spite of the continuation of sulfanilamide therapy, increasing numbers of pneumococci reappeared in the sputum. This is exemplified by Cases C.C., F.S., W.L., C.P., and J.A. in Table II. The above findings would seem to indicate that the pneumococci, once sensitive, had become refractory to the drug.

TABLE II

CASE	TYPE	DAYS ONSET TO B	BLOOD CULTURE	COUNT BEFORE B	SPUTUM COUNTS AFTER THERAPY WAS STARTED WITHIN									
					12°	24°	48°	72°	96°	120°	144°	168°	192°	
Serum B	O.G.	3	-	35	24	22*	13	1	oc	oc				
	C.T.	2	+	31	15	20*	15	9	7					
	J.I.L.	3	-	26	11*	15	8	oc	oc					
	F.B.	7	-	32	42*	11	12	1	oc					
	R.B.	2	-	8	17	7	1	oc	oc					
Sulfanilamide B	D.F.	1	+	12	<1	oc	oc	oc	oc*					
	F.D.	1	+	21	20	1*	<1	oc						
	E.E.	3	-	19*	oc	oc	oc	oc						
	D.C.	2	-	17	1	oc								
	G.C.	2	-	3	oc	oc	oc	oc	1	1	3*	oc	oc	
Sulfanilamide B	F.S.	2	-	3	5	oc	oc	oc	oc	1	7*	oc	oc	
	W.L.	2	+	21	15	1	oc	oc	1	1*	2	<1	8	
	C.P.	2	+	53	45	5	8	28*	28	14	4	<1	<1	
	J.A.	2	+	5	5	<1	oc	12	15	38*	<100>	Died	<1	
	M.W. Co.P.	2	?	41	30	27	20	20*	13	10	<1	3		
	IV	2	-	35	9	17	10	20*	15	15	--			

\*Represents the day on which clumping was first noted  
 Oc represents an occasional pneumococcus seen.

Only 2 of the 59 recovered cases treated with sulfanilamide (M.W. and Co.P.) failed to show a bacteriostatic effect in the sputum within thirty-six hours. M.W. did not develop adequate blood sulfanilamide levels until the sixth hospital day, but in the case of Co.P., 12.8 mg. per cent were obtained on the second hospital day. Nevertheless, only a transitory sulfanilamide effect was noted in the sputum.

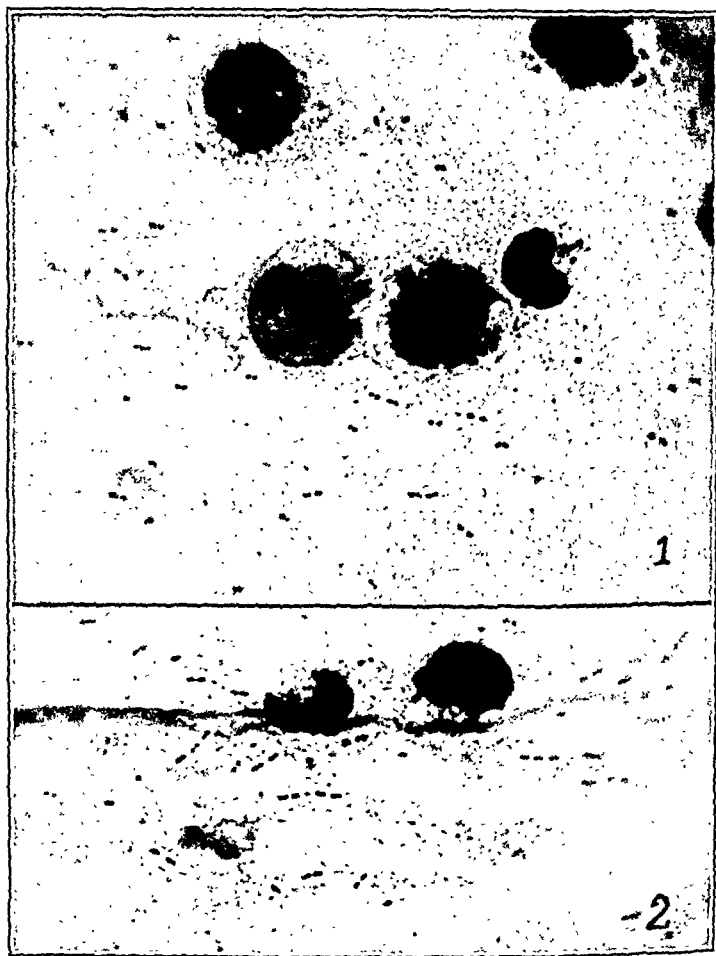


Fig. 1.—Sputum specimen before therapy. Note the number of pneumococci per field, absence of clumping, and phagocytosis.

Fig. 2.—Specimen eight hours after sulfanilamide therapy was begun. Note that the pneumococci have not decreased but are chained, irregularly shaped, and some poorly stained.

The possible role of the immunity mechanism in its relation to sulfanilamide therapy must also be considered. In all except one of the cases presented in Table II (D.C.), clumping of extracellular encapsulated pneumococci was observed at some time during the course of the disease, but this phenomenon had no relation to the dose or period of administration of sulfanilamide. It is interesting to note that those patients who developed clumping during the first four days of treatment (D.F., F.D., E.E.) were the ones who responded best to the drug. The remaining patients who failed to show clumping before the fifth to the seventh hospital day were also the ones in whom the pneumococci returned to the sputum following the good initial drug effect. Case J.A. became refrac-

tory to sulfanilamide on the fourth hospital day. The pneumococci increased progressively thereafter, and, although he developed early clumping, the organisms multiplied so rapidly that he died on the evening of the seventh hospital day. Cases M.W. and Co.P. showed clumping in the sputum on the fourth and sixth hospital days. This was followed by a gradual decrease in number of free pneumococci, together with a marked increase in phagocytosis, similar to that seen in serum-treated patients. The correlation between the sputum findings and the clinical response of the patient will be reported in a separate communication when a larger series of cases has been studied.

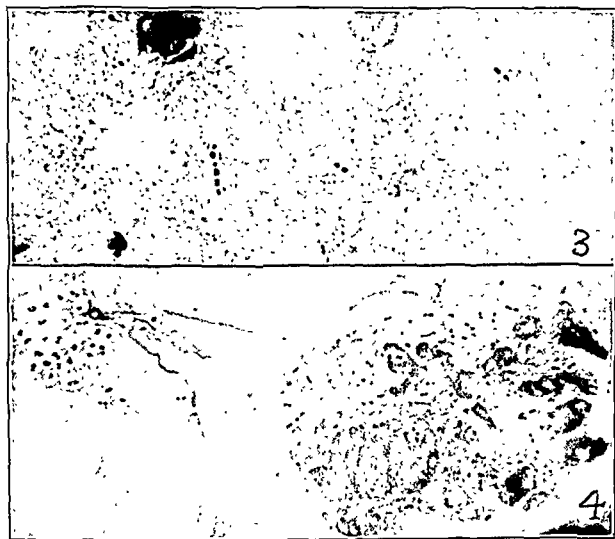


Fig. 3.—Specimen thirty-two hours after sulfanilamide therapy was begun. Note the marked reduction in pneumococci. The chaining and irregular staining character persist. The apparent decrease in capsule size is due to the thickness of the smear rather than to any actual reduction.

Fig. 4.—Specimen eighty hours after sulfanilamide therapy was begun. Note the return of pneumococci in large number, clumping of extracellular organisms, and associated increased phagocytosis.

The relationship between the initial bacteriostatic effect of sulfanilamide, the reappearance of pneumococci in the sputum, and the onset of clumping of extracellular organisms may best be illustrated by the patient C.P. (Table II). He was a colored male, aged 25, who was admitted to the hospital on the third day of illness. The blood culture contained 6 type II pneumococci per cubic centimeter, roentgen and clinical evidences of consolidation of the right lung field were present, and his total leucocyte count was only 7,300. Treatment with sulfanilamide was begun fifty-five hours after the onset of symptoms. He received a total of 36 gm. of sulfanilamide over a five-day period, with blood levels varying from 5.6 to 7.7 mg. per cent. The drug was discontinued on the fifth hospital day because the patient developed jaundice.

Sputum examination before therapy was instituted showed 53 pneumococci per oil immersion field, 1 or 2 pneumococci per phagocyte, and no evidence of clumping of extracellular organisms (Fig. 1). Eight hours after the initiation of sulfanilamide therapy, the sputum contained over 50 pneumococci per field, but the organisms were chained and assumed bizarre shapes (Fig. 2). Twenty-four hours later the maximum drug effect was noted, and the extracellular pneumococci dropped to 5 per field (Fig. 3). Bacteriostasis was maintained throughout the next day, but on the morning of the fifth hospital day the extracellular pneumococci began to return to the sputum, and the count rose to 22 per field. By evening it had reached 33, and definite clumping was noted for the first time. On the morning of the sixth day clumping and phagocytosis had increased (Fig. 4), and thereafter a progressive reduction in the number of free pneumococci took place (Table II). The patient made a gradual but uneventful recovery.

#### DISCUSSION

If the sputum findings reflect the actual pneumonic process in the lung, then the results of this study lend strong support to the concept that sulfanilamide is of value in the treatment of pneumococcal pneumonia.<sup>3</sup> The bacteriostatic effect, as indicated by the rapid fall in number of pneumococci per field in the sputum, is in harmony with what is known concerning the action of the drug in vitro and in vivo.<sup>4-11</sup> The mode of action of sulfanilamide and related compounds, however, is still open to speculation. Changes in the capsule have been suggested by some,<sup>10, 12-14</sup> but the claims have not been substantiated by others.<sup>11, 15, 16</sup> Increased susceptibility to phagocytosis has been noted, particularly for streptococci, by Long, Bliss, and others.<sup>4, 17</sup> Recently Long, Bliss, and Feinstone<sup>16</sup> were unable to show an increased phagocytosis in sulfanilamide-treated mice infected with types I, II, and III pneumococci. Disintegrative and morphologic changes in streptococci<sup>18, 19</sup> and pneumococci<sup>16</sup> have also been described. As already mentioned, no evidence has as yet been obtained during this study to show that sulfanilamide induces changes in the capsule of the pneumococci or that it renders them more susceptible to phagocytosis. These findings, together with the minor changes in morphology and staining character of the pneumococci, are in accord with those of Long and others.<sup>16, 18, 19</sup>

Recently Lawrence,<sup>21</sup> and Telling and Oliver<sup>24</sup> described a decrease in the number of organisms in the sputum during sulfapyridine therapy. They also noted that the Quellung reaction disappeared. However, Fleming,<sup>22</sup> McIntosh and Whitby,<sup>23</sup> and Long<sup>24</sup> were unable to substantiate the loss of type specificity in infected animals under treatment with the drug. We have also attempted to repeat the experiments of Lawrence, Telling, and Oliver in sulfanilamide-treated cases. At times during therapy a diligent search was necessary, but when encapsulated pneumococci were found in the sputum, they gave the typical Neufeld reaction.

The reappearance of the pneumococci in the sputum after three to five days suggested that the organisms, which were still typeable, had become refractory to the action of sulfanilamide. This fact must be considered in the evaluation of therapy because of its possible influence upon the clinical course of the pneumonia. For example, one might expect that a patient whose sputum con-

tained clumps of encapsulated pneumococci would respond very differently from one who had become refractory to sulfanilamide and had developed clumping on the seventh hospital day. Boak and Carpenter<sup>25</sup> have recently shown that gonococci cultivated on media containing sublethal doses of sulfanilamide were then able to survive lethal concentrations of the drug. Lyons<sup>17</sup> has reported a fatal case of hemolytic streptococcus septicemia, treated with sulfanilamide, in which the organisms were found to be resistant to phagocytosis. Whitby<sup>20</sup> has also suggested the possibility of the bacteria's becoming refractory and advises that therapy be continued with another related drug.

The clumping of encapsulated pneumococci in the sputum of sulfanilamide-treated cases at some time during the course of the disease, would seem to indicate that the development of active immunity also plays a role in the outcome of the pneumonia.<sup>2, 3</sup> McIntosh and Whitby<sup>23</sup> have made some analogous observations on mice who recovered from pneumococcal infection after treatment with sulfapyridine. They found that these animals were protected from reinfection in the same way as actively immunized mice and suggest that the immunity, and not the previous drug therapy, was responsible for the resistance. However, Long<sup>24</sup> was unable to substantiate the above results. According to the findings in the sputum, the value of sulfanilamide in the treatment of pneumonia seems to depend upon its limited ability to inhibit the growth of the pneumococci during that period of time necessary for the patient to develop his own immune response; or to permit a partially immune but overwhelmed host to mobilize his defensive forces effectively.

#### SUMMARY

1. The administration of sulfanilamide to patients with pneumonia caused a decrease of encapsulated pneumococci in the sputum within twenty-four to thirty-six hours.
2. In some cases, the bacteriostatic effect was maintained for two to four days, at which time the pneumococci again returned to the sputum.
3. The relationship between the effect of sulfanilamide and the appearance of clumps of extracellular pneumococci in the sputum is discussed.

*Addendum.*—A bacteriostatic effect analogous to that produced by sulfanilamide has also been observed in the sputum of patients treated with sulfapyridine. However, resistance to the drug, as judged by the return of pneumococci to the sputum, has not as yet been noted.

Photographs by C. G. Eddy, Eloise Hospital, Eloise, Mich. (Magnification 1,800.)

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## THE DETERMINATION OF pH VALUES OF BIOLOGIC FLUIDS. I\*

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### INTRODUCTION

MANY laboratories using sera, media, infusions, etc., determine pH values of such materials as a routine procedure. Colorimetric methods are employed, as a rule, probably because of the comparatively rapid and convenient techniques which have been developed. However, different laboratories have developed somewhat different procedures for the electrometric and colorimetric methods. In this paper we present a comparative experimental study of the pH values obtained for various buffer solutions and the biologic products and bacteriologic media in this laboratory with the routine colorimetric procedures in use here, and with the electrometric method using the glass electrode. Other laboratories may use still different methods or modifications of some given methods.

In the practical application of any method for pH determinations (whether electrometric or colorimetric) it is well to recall that the successful application of a given procedure justifies its use. At the same time, the knowledge of the pH values of test materials based upon some standard reference electrode system is of interest and importance if only for comparison with the work of others.

### EXPERIMENTAL METHODS

The colorimetric methods were as follows:

- I. Hellige color disk comparator method.<sup>†</sup>
- II. Dilution method based on Sørensen's standard buffer mixtures and indicators.<sup>1</sup>
  1. Sørensen phosphate buffer mixtures.
  2. Walpole acetate buffer mixtures.
  3. Clark and Lubs borate buffer mixtures.

In method I a standard Hellige hydrogen-ion comparator<sup>†</sup> was used.

In method II a wooden "block comparator" of the type described by Hurwitz, Meyer, and Ostenberg<sup>2</sup> was used.

\*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.

<sup>†</sup>Hellige hydrogen-ion comparator standard color disks and indicator solutions prepared by Hellige, Inc.

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The following indicators were used in the colorimetric determinations:

I. HELLIGE COLOR DISK COMPARATOR METHOD*			II. MODIFIED SØRENSEN DILUTION METHOD		
INDICATOR	pH RANGE	CONCENTRATION %	INDICATOR	pH RANGE	CONCENTRATION %
a. Phenol red	6.8-8.4	0.04 (W)	a. Phenol red	6.8-8.4	0.02 (W)
b. Bromthymol blue	6.0-7.6	0.04 (W)	b. Bromthymol blue	6.0-7.6	0.04 (A)
c. Meta-nitrophenol	6.8-8.4	0.3 (W)	d. Thymol blue	8.0-9.6	0.04 (A)
j. Amino red	6.8-8.0	**	e. Bromocresol purple	5.2-6.8	0.04 (A)
k. Gamma-dinitrophenol	4.0-5.4	0.025 (W)	h. Methyl red	4.4-6.0	0.02 (A)
l. Para-nitrophenol	5.4-7.0	0.1 (W)			

W = distilled water.

A = alcohol.

\*\*Concentration and solvent not given by Hellige, Inc.

The electrometric method included the MacInnes-Beleher glass electrode-saturated calomel electrode in conjunction with the Young electron ray meter.<sup>6</sup> By keeping the glass electrode filled with distilled water when not in use, the residual potential within the glass membrane (asymmetric potential) was maintained at a constant value. The MacInnes-Beleher glass electrode has been amply described in the literature.<sup>3-5, 7</sup>

The Young electron ray meter is constructed to record directly the electromotive force or the pH values. This pH meter employs a cathode-ray tube in conjunction with a five-element vacuum tube as null-point indicator.

MacInnes and his co-workers have recently made accurate determinations of the pH values of a number of buffer solutions.<sup>6</sup> Employing a quadrant electrometer in conjunction with the hydrogen and glass electrodes, they found the following readings for 0.05 molar acid potassium phthalate ( $\text{KHC}_8\text{H}_4\text{O}_4$ ):

pH  $4.000 \pm 0.005$  at  $12^\circ \text{C}$ .

pH  $4.000 \pm 0.005$  at  $25^\circ \text{C}$ .

pH  $4.015 \pm 0.005$  at  $38^\circ \text{C}$ .

The temperature coefficient of this solution is approximately zero between  $12^\circ$  and  $25^\circ \text{C}$ ., and about 0.001 pH per degree between  $25^\circ$  and  $38^\circ \text{C}$ . Since measurements with the MacInnes-Beleher glass electrode of a variety of buffer solutions have demonstrated a linear relationship between pH and electromotive force<sup>7</sup> from pH 1 to pH 9, 0.05 molar acid potassium phthalate is well suited as a calibrating and reference solution. The electrode was also regularly checked by means of a borate buffer solution of pH 8.00.

#### EXPERIMENTAL RESULTS

Data will be presented for (a) buffer solutions, (b) bacteriologic media, and (c) biologic products.

In using the glass electrode assembly, the pH value of the test solution may be obtained by direct observation on the electron ray meter. It may also be calculated from the electromotive force observed by means of the usual equation, using 0.05 M acid potassium phthalate as reference solution.<sup>7</sup> Since the two sets of values so obtained differed by less than 0.02 pH except for 2 cases in which the difference was 0.03, only the former readings will be presented in the tables.

\*Built by S. B. Young, now with the American Instrument Co.

Whenever the solutions for either colorimetric or electrometric study were diluted, it is so stated in the tables. In all other cases the solutions and mixtures were used directly as prepared and described.

The pH of the water used for dilution was found by the glass electrode, after standing for several hours at room temperature and exposed to the air, to range from 6.28 to 6.32 for different samples. The saline varied from pH 6.27 to 6.35.

A number of results obtained with buffer solutions are presented in Table I. These solutions were prepared as described by Clark. In the first part of the table the effects on the glass electrode measurements of dilution with water and with saline (0.85 per cent sodium chloride solution) are shown in columns 2 and 3. In the colorimetric estimations an indicator is naturally added. The changes in pH due to the presence of indicator substances were measured, and the pH values are shown in columns 1, 2, and 3. The indicators used are given in the last column of the table. The colorimetric results obtained with the Hellige color disk comparator for the three sets of solutions, undiluted, diluted with water, and diluted with saline, are presented in columns 7, 8, and 9. The volume for each measurement was 3.5 c.c. made for the dilutions as follows:

Column 1—Undiluted solution.

Columns 4 and 7—3 c.c. solution and 0.5 c.c. indicator solution.

Column 2—1 c.c. solution and 2.5 c.c. water.

Column 3—1 c.c. solution and 2.5 c.c. saline

Columns 5 and 8—1 c.c. solution, 2 c.c. water, and 0.5 c.c. indicator solution.

Columns 6 and 9—1 c.c. solution, 2 c.c. saline, and 0.5 c.c. indicator solution.

In the second part of the table the glass electrode method and the modified Sørensen dilution colorimetric method, using the comparator block of Hurwitz, Meyer, and Ostenberg,<sup>2</sup> were compared for a number of the buffer solutions undiluted and diluted with four volumes of distilled water. For the colorimetric readings with these solutions, 0.25 c.c. indicator was added to 2 c.c. of undiluted test fluid and diluted to 10 c.c. with distilled water.

The results shown in Table I may be summarized as follows:

In the glass electrode measurements, both in the absence and in the presence of indicator substances, dilution with water increased, and dilution with saline decreased the pH. The most alkaline phosphate mixture tested was the only exception. Most of the differences between the original and diluted solutions were less than 0.2 pH. The presence of the indicator made very little difference in the readings; only in a few cases was this difference greater than 0.05 pH. In the readings with the Hellige comparator and disks, possibly 0.05 pH would represent the reliability of the color comparisons by an experienced worker. The readings of the water-diluted and saline-diluted solutions agreed and disagreed with each other with no apparent regularity. The largest difference was 0.3 pH; a number were 0.2 pH. Similar differences existed with the undiluted colorimetric solutions. In a comparison with the glass electrode measurements, the variations in the colorimetric readings, as a rule, lie within 0.2 pH or less. Whether this is to be considered large depends on the solution in question. For

TABLE 1

PH VALUES OF BUFFER SOLUTIONS BY THE GLASS ELECTRODE AND COLORIMETRIC METHODS

Part 1.  $t. = 25^{\circ} \text{C.}$ 

GLASS ELECTRODE			GLASS ELECTRODE			HELMGE COLORIMETER			
UNDI- LUTED (1)	1 VOL. + 2.5 VOL. WATER (2)	1 VOL. + 2.5 VOL. SALINE (3)	3 VOL. + 0.5 VOL. INDI- CATOR (4)	1 VOL. + 2 VOL. WATER + 0.5 VOL. INDI- CATOR (5)	1 VOL. + 2 VOL. SALINE + 0.5 VOL. INDI- CATOR (6)	3 VOL. + 0.5 VOL. INDI- CATOR (7)	1 VOL. + 2 VOL. WATER + 0.5 VOL. INDI- CATOR (8)	1 VOL. + 2 VOL. SALINE + 0.5 VOL. INDI- CATOR (9)	INDI- CATOR USED (10)
<i>Phthalate Buffer Solutions</i>									
4.07	4.19	4.00	4.09	4.22	4.02	3.9s	4.0s	4.0s	(k)
4.23	4.33	4.10	4.26	4.36	4.15	4.1s	4.2s	4.2s	(k)
4.43	4.53	4.29	4.46	4.57	4.33	4.3s	4.4s	4.4s	(k)
4.65	4.78	4.49	4.69	4.80	4.55	4.6s	4.6s	4.6s	(k)
4.84	4.97	4.67	4.87	4.97	4.72	4.8s	4.8s	4.8s	(k)
5.07	5.19	4.90	5.11	5.21	4.95	5.0s	5.0s	5.0s	(k)
5.27	5.38	5.14	5.30	5.40	5.17	5.2s	5.2s	5.1s	(k)
5.47	5.60	5.33	5.50	5.60	5.38	5.4s	5.4s	5.4s	(k)
5.69	5.82	5.55	5.73	5.84	5.60	5.7s	5.8s	5.6s	(l)
5.90	6.03	5.75	5.95	6.05	5.81	6.0s	6.0s	5.9s	(l)
6.08	6.21	5.91	6.08	6.21	5.95	6.0s	6.2s	6.0s	(l)
6.39	6.49	6.15	6.39	6.46	6.20	6.4s	6.5s	6.4s	(l)

<i>Phosphate Buffer Solutions</i>									
5.83	6.00	5.65	5.84	5.96	5.64	5.8s	5.9s	5.8s	(l)
6.00	6.14	5.80	6.03	6.15	5.85	6.0s	6.0s	6.0s	(l)
6.19	6.34	6.02	6.22	6.34	6.06	6.2s	6.2s	6.2s	(l)
6.40	6.54	6.25	6.42	6.54	6.28	6.4s	6.5s	6.4s	(l)
6.59	6.74	6.46	6.61	6.72	6.49	6.7s	6.8s	6.6s	(l)
6.81	6.95	6.68	6.83	6.92	6.69	6.9s	7.0s	6.8s	(l)
6.98	7.12	6.85	7.02	7.15	6.87	6.9s <sup>(11)</sup>	7.0s	7.0s	(c)
7.20	7.32	7.07	7.24	7.32	7.14	7.2s	7.4s	7.3s	(c)
7.41	7.52	7.28	7.44	7.67	7.31	7.4s	7.5s	7.3s	(c)
7.59	7.85	7.45	7.62	7.74	7.47	7.5s	7.6s	7.4s	(c)
7.73	7.84	7.56	7.72	7.74	7.53	7.7s	7.6s	7.5s	(c)
7.94	8.09	7.76	7.93	7.89	7.69	7.8s	7.8s	7.6s	(c)

Part 2.  $t. = 25^{\circ} \text{C.}$ 

GLASS ELECTRODE		COLORIMETRIC-MODIFIED SÖRENSEN DILUTION METHOD			GLASS ELECTRODE		COLORIMETRIC-MODIFIED SÖRENSEN DILUTION METHOD		
UNDI- LUTED (1)	1 VOL. + 4 VOL. WATER (2)	1 VOL. + 0.25 VOL. INDI- CATOR (3)	1 VOL. + 4 VOL. WATER + 0.25 VOL. INDI- CATOR (4)	INDI- CATOR USED (5)	UNDI- LUTED (1)	1 VOL. + 4 VOL. WATER (2)	1 VOL. + 0.25 VOL. INDI- CATOR (3)	1 VOL. + 4 VOL. WATER + 0.25 VOL. INDI- CATOR (4)	INDI- CATOR USED (5)
<i>Phthalate Buffer Solutions</i>					<i>Phosphate Buffer Solutions</i>				
4.24	4.39	4.0s	4.2s	(h)	5.84	6.02	5.8s	5.8s	(e)
4.23	4.59	4.2s	4.4s	(e)	5.98	6.15	5.9s	6.0s	(e)
4.65	4.81	4.4s	4.8s	(h)	6.40	6.56	6.3s	6.2s	(e)
4.83	5.02	4.7s	4.9s	(h)	6.78	6.95	6.7s	6.8s	(h)
5.88	6.08	5.9s	5.9s	(e)	7.63	7.78	8.0s	8.0s	(a)
6.07	6.21	6.0s	6.1s	(e)	<i>Borate Buffer Solutions</i>				
6.38	6.57	6.3s	6.5s	(e)	7.69	7.80	7.6s	7.6s	(2)
					8.14	8.21	8.2s	8.2s	(d)
					8.51	8.62	8.6s	8.5s	(d)
					8.71	8.73	8.8s	8.8s	(d)
					8.93	9.00	9.1s	9.0s	(d)
					8.98	9.04	9.1s	9.0s	(d)

TABLE II

PH VALUES OF BACTERIOLOGIC MEDIA BY THE GLASS ELECTRODE AND COLORIMETRIC METHODS

MATERIAL (1)	T ° C. (2)	GLASS ELECTRODE		HELLIGE COLORIMETRIC		COLORIMETRIC-MODIFIED SØRENSEN DILUTION METHOD		
		INDI- LUTED (3)	DILUTED 1 VOL. + 4 VOL. WATER (4)	5 VOL. + 0.25 VOL. INDI- CATOR (5)	INDI- CATOR USFD (6)	10 VOL. + 0.25 VOL. INDI- CATOR (7)	2 VOL. + 8 VOL. WATER + 0.25 VOL. INDI- CATOR (8)	INDI- CATOR USED (9)
Beef heart infusion	25°	5.87	6.03	-	-	5.71	5.71	(c)
		6.10	6.26	6.01	(b)	6.01	6.01	(e)
		6.32	6.43	6.01	(b)	6.01	6.01	(e)
	30°	5.98	6.13	5.91	(b)	5.91	6.01	(e)
		6.04	6.22	6.01	(b)	5.91	6.01	(e)
Pneumococcus broth	25°	6.05	6.23	5.91	(b)	5.91	6.01	(e)
		7.76	7.81	7.81	(a)	7.61	7.61	(a)
		7.85	7.92	7.81	(a)	7.81	7.81	(a)
	30°			7.51	(j)			
		7.68	7.76	7.81	(a)	7.61	7.61	(a)
				7.41	(j)			
		7.85	7.91	7.91	(a)	7.81	7.81	(a)
				7.51	(j)			
		7.89	7.95	7.91	(a)	8.01	7.91	(a)
		7.78	-	7.91	(a)	7.91	7.91	(a)
		7.55	7.63	7.81	(a)	7.41	7.41	(a)
		7.76	7.81	7.91	(a)	7.81	7.81	(a)
Beef infusion	25°	6.11	6.28	-	-	6.01	6.01	(e)
		6.69	6.81	6.41	(b)	6.51	6.51	(e)
		6.24	6.37	6.01	(b)	6.11	6.01	(e)
Veal infusion	25°	6.51	6.62	-	-	6.31	6.31	(e)
		6.97	7.09	6.91	(a)	6.81	6.81	(b)
				6.81	(b)			
				6.81	(j)			
		6.16	6.31	6.01	(b)	6.01	6.01	(e)
Beef heart broth	30°	7.04	7.16	7.21	(b)	-	7.21	(a)
		7.52	7.55	7.81	(a)	7.71	7.61	(a)
Diphtheria broth	25°	7.33	7.37	7.61	(a)	7.21	7.21	(a)
				7.21	(b)			
				7.11	(j)			
Phosphate broth	25°	7.02	7.08	6.81	(b)	6.81	6.81	(b)
		7.81	7.85	7.91	(a)	7.71	7.71	(a)
		7.94	7.99	7.81	(a)	7.91	7.91	(a)
				7.51	(j)			

many biologic fluids such differences might not be considered serious. At the same time the possibility of such divergence must be kept in mind.

Similar conclusions hold for the results in the second part of the table. Dilution with water caused similar changes in the measurements. The colorimetric readings for the phthalate and phosphate buffer solutions, as a rule, were less than the electrometric, and for the borate buffer solutions were greater than the electrometric. The differences between the colorimetric and electrometric results were less than 0.3 pH except for a few solutions.

A number of results obtained by methods in use at the William Hall Park Laboratory with bacteriologic media are presented in Table II. Here also, diluting the media with water gave increased pH values with the glass electrode measurements. The colorimetric method, on the other hand, showed little differences between the diluted and undiluted solutions. A comparison of the two colorimetric methods with the glass electrode method did not show regular differences. In many cases the results agreed closely; in a number there were divergences. The differences were not greater than 0.3 pH. While this may seem large, it occurred only occasionally, so that, while the possibility of a difference as large as this must be kept in mind, in many measurements the colorimetric results obtained as described in this table agree satisfactorily with the glass electrode results.

TABLE III  
pH VALUES OF BIOLOGIC PRODUCTS BY THE GLASS ELECTRODE AND COLORIMETRIC METHODS  
t. = 25° C.

t. = 25° C.

PRODUCT	UNDILUTED GLASS ELECTRODE	HELLIGE COLORIMETRIC	
		1 VOL. + 2 VOL. SALINE + 0.5 VOL. INDICATOR	INDICATOR USED
<i>Pneumococcus Antibacterial Preparations</i>			
Type I	6.95	6.9 <sub>s</sub>	(1)
	7.00	6.9 <sub>s</sub>	(1)
Type V	6.98	7.0 <sub>s</sub>	(1)
	6.94	6.9 <sub>s</sub>	(1)
Type VI A and B	6.50	6.4 <sub>s</sub>	(1)
	6.81	6.9 <sub>s</sub>	(1)
Type XVIII		6.9 <sub>s</sub>	(c)
	6.81	6.8 <sub>s</sub>	(1)
		6.8 <sub>s</sub>	(c)
<i>Pneumococcus Antibacterial Sera Before Dialysis</i>			
Type IV	7.87	7.4 <sub>s</sub>	(c)
Type V	7.30	7.2 <sub>s</sub>	(c)
Type VII	7.40	7.4 <sub>s</sub>	(c)
<i>After Dialysis</i>			
Type I	7.48	7.3 <sub>s</sub>	(c)
Type IV	7.59	7.4 <sub>s</sub>	(c)
Type V	7.39	7.3 <sub>s</sub>	(c)
Type XIV	7.25	7.0 <sub>s</sub>	(1)
Type XVIII	7.50	7.4 <sub>s</sub>	(c)

PRODUCT	UNDILUTED GLASS ELECTRODE	HELLIGE COLORIMETRIC	
		1 VOL. + 2 VOL. SALINE + 0.5 VOL. INDICATOR	INDICATOR USED
<i>Pneumococcus Antibacterial Serum at Various Stages of the Refining Process</i>			
Type I	7.48	7.3 <sub>s</sub>	(c)
Type II	5.07	5.1 <sub>s</sub>	(k)
	5.03	5.0 <sub>s</sub>	(k)
	4.96	5.1 <sub>s</sub>	(k)
	5.85	5.9 <sub>s</sub>	(k)
	5.03	5.2 <sub>s</sub>	(k)
	5.93	5.9 <sub>s</sub>	(1)
Type IV	5.53	5.9 <sub>s</sub>	(1)
Type VII	5.12	5.1 <sub>s</sub>	(k)
Type VIII	5.62	5.8 <sub>s</sub>	(1)
Type XIV	4.97	5.1 <sub>s</sub>	(k)
Type XVIII	5.09	5.1 <sub>s</sub>	(k)
	5.68	5.7 <sub>s</sub>	(1)
Normal horse serum	7.26	7.3 <sub>s</sub>	(c)
	7.34	7.1 <sub>s</sub>	(c)
Antiscarlatalin serum after dialysis	5.76	5.9 <sub>s</sub>	(1)
Antiscarlatalin serum during refining process	6.70	6.7 <sub>s</sub>	(1)
	5.65	5.9 <sub>s</sub>	(1)

The results

The results obtained with 35 samples of biologic products are presented in Table III. Comparisons of the glass electrode (undiluted solutions) and Hellige colorimetric (with saline dilution only) were made. The agreements of the pH values were remarkably close in most cases. The striking exceptions were with Type IV pneumococcus antibacterial preparations, where three measurements of the serum material at different stages of the refining process gave differences in

pH values of 0.42, 0.19, and 0.37. Two other preparations of different pneumonia type antibodies gave differences of 0.25 and 0.23 pII unit, one normal horse serum difference was 0.24 pII and one antiscarlatinal serum difference was 0.30 pII.

#### DISCUSSION

The pH of a solution is a function of its hydrogen-ion activity. The activity of an individual ion species cannot, however, be obtained in any simple unequivocal way. If measured by electrometric methods, certain assumptions are introduced in calculating the pII values from the electromotive forces which are measured. If colorimetric methods are used, color changes involving tautomeric forms of organic substances are involved. Electromotive force measurements and tautomeric changes may be considerably influenced by other substances present in solutions. These problems have been frequently discussed and studied. It will suffice to mention these facts without entering into the details.

In view of these relations, the question of what would be the most probable pII value of a solution must be considered. At the present time, the values obtained from the electromotive forces by means of the glass electrode are readily reproducible. They are based, in the customary way, upon the normal hydrogen electrode as the standard reference electrode. For most of the test solutions for which results are presented in this paper, the agreement between the electrometric and colorimetric methods was well within  $\pm 0.3$  pII unit. Within these limits any one of the colorimetric methods outlined above is satisfactory, provided color standards are checked periodically by means of some standard electrode system. A factor to be considered seriously is the effect of dilution. Practically all the colorimetric methods for determining the pII values of biologicals involve dilution with water or saline. These dilutions change the pH, as shown in the experimental results. In our solutions dilution with water increased the pH and dilution with saline decreased the pII. The diluted solutions gave similar results by the electrometric and colorimetric methods. It is evident that the pH values of these diluted solutions were different from the pH values of the undiluted solution, the differences with some of these solutions amounting to 0.2 pH. This fact must be taken into account in considering that the biologicals as actually used differ somewhat from the values given. Furthermore, the colorimetric measurements should always be made under identical conditions with respect to volume of test solution, volume, and character of diluent, temperature, etc. Even when these factors are kept constant, there may be marked differences between the electrometric and colorimetric methods due to specific effects of the test fluid.

#### SUMMARY

An experimental study of the pH values of buffer solutions, bacteriologic media, and biologicals is presented, using the glass electrode and some of the colorimetric procedures which have been developed. The differences in the pH values obtained under the different conditions of testing and the possible influence of these differences in routine work are indicated.

Thanks are due Dr. Jacob Weinberg and Mr. Eugene Caidone for their colorimetric determinations of the test fluids.



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## A FURTHER NOTE ON THE STABILITY OF GLYCERINATED ANTISHEEP HEMOLYSIN\*

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IN 1924 I reported the satisfactory preservation of glycerinated antisheep hemolysin for a period of seven years.<sup>1</sup> It was then shown that by the addition of an equal volume of glycerin, as advocated by Kolmer<sup>2</sup> and Clock and Beard,<sup>3</sup> this hemolysin, though subjected to varying and even marked changes of temperature over varying periods of time, having had an original titer of 1:20,000, after seven years still had a titer of 1:16,000 with 0.5 c.c. of 1:30 complement and 0.5 c.c. of 2 per cent sheep cell suspension.

An ampoule of this same lot of hemolysin made in 1917 was recently unearthed and again titered twenty-two years after its preparation. With a dose of 0.5 c.c. of 2 per cent sheep cell suspension and 0.5 c.c. of 1:30 lyophile complement prepared after the method of Flosdorf and Mudd, a titer of 1:3,000 was obtained.

While this evidences a marked decrease from the original titer of 1:20,000, it suffices to demonstrate that glycerinated antisheep amboceptor for use in complement fixation tests is remarkably stable and can be preserved satisfactorily for extended periods. It also emphasizes, incidentally, that anomalous results in complement fixation tests are never due to failure or depreciation of antisheep hemolysin.

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## THE TREATMENT OF BENIGN PROSTATIC HYPERTROPHY WITH TESTOSTERONE PROPIONATE\*

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ENDOCRINOLOGY has made rapid strides within the past decade. Its benefits have been well utilized by the gynecologists in the treatment of innumerable disorders of the female. Practical endocrinology in the male has been neglected, aside from the early attempts to treat impotence and senescence.

Brown-Séquard, administering testicular extract to himself, alleged that his vigor and capacity for work were increased. Voronoff and Steinaeh applied this principle of rejuvenation by transplanting testes of apes into human beings. These theories were discredited when it became evident that testicular atrophy had no causal relationship to senescence. More recently, endocrinologists,<sup>1, 2</sup> impressed with the fact that the normal condition of the prostate is dependent upon an intact pituitary-testes relationship, have initiated researches and clinical experiments designed to illuminate the baffling problem of the etiology and treatment of benign prostatic hypertrophy.

The following two theories, both depending upon a disproportionate relationship between the sex hormones, are currently expounded in explanation of the etiology of benign prostatic hypertrophy:

1. The testes produce two hormones—one by the germinal epithelium of the seminiferous tubules, and the other by the interstitial cells (cells of Leydig). From puberty to the age of 50 the first hormone has an inhibitory effect on the production of gonadotropic hormone by the anterior pituitary, which in turn prevents the production of the second hormone, the male sex hormone, which is responsible for prostatic hypertrophy. At about 50 there is a lessened secretion of the germinal epithelium, removing the pituitary inhibition and permitting overactivity of the cells of Leydig with overproduction of testosterone and consequent prostatic hypertrophy. This theory is held by Martins and Rocha<sup>3</sup> and by McCullagh and Walsh.<sup>4</sup>

2. Male and female sex hormones are present in both sexes in certain proportions throughout life. In males, as age progresses there is a gradual diminution in the production of male sex hormone, whereas the amount of circulating female sex hormone remains relatively constant. Hence, the diminution of the male sex hormone permits a predominant activity of the female sex hormone which results in hypertrophy of the prostate. This theory is held by de Jongh<sup>5</sup> and Laqueur.<sup>6</sup>

Biologic methods of experimentation, presently at our disposal, seem to favor the second theory which ascribes the prostatic hypertrophy to the unan-

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tagonized action of the female sex hormone, estrogen. The following data may be marshaled in support of this theory:

1. *Animal Experimentation*.—Lacassagne<sup>7</sup> in 1933 described prostatic metaplasia and stratification of the urethral epithelium, with subsequent narrowing of the urethra and dilatation of the bladder of mice that had received injections of crystalline estrogen. Burrows and Kennaway,<sup>8</sup> after applying estrogen to the skin of mice, obtained a distention of the prostatic alveoli which became lined by keratinized squamous epithelium. They called attention to the similarity of the histologic picture, the accompanying urinary dribbling, hydroureter, and hydronephrosis seen in this experimentally produced condition, to the syndrome of benign enlargement of the prostate in man. Zuckerman<sup>9</sup> produced prostatic hypertrophy in the immature rhesus monkey within fourteen days by daily injections of 100 gamma. Zuckerman observed enlargement of the prostate, relative diminution of the prostatic glands, and stratification of the columnar epithelium. These alterations could be inhibited by the simultaneous administration of a pure preparation of male sex hormone or, when developed, could be eradicated by its administration.

Similar experiments in mice by numerous investigators yielded results comparable to those of Zuckerman. Ruseh,<sup>10</sup> employing male mice, administered subcutaneously injections of estrogen (progynon B) twice weekly for a period of from 122 to 137 days. Pathologic changes of the prostate were noted within thirty to seventy days. They included a change from columnar to stratified squamous epithelium and an increase in the fibromuscular stroma. Shortly afterward, dilatation of the urinary bladder and development of serotal hernias uniformly appeared. With the establishment of these alterations there was an actual, though slight, decrease in the size and weight of the prostate. Administration of the male sex hormone, testosterone, induced a reversal of the stratified squamous epithelium to the columnar epithelium, greater secretion of the mucous glands, and increase in the weight of the prostate. The testosterone did not reduce the augmented fibromuscular stroma nor the size of the dilated bladder. When testosterone was employed early in the experiment, it delayed the appearance of the dilated bladder from forty to fifty days. As control substances Ruseh employed progesterone and dehydroandrosterone, but neither of these produced reversal of the epithelial metaplasia.

While it appears from these experiments that testosterone causes enlargement of the prostate, Ruseh cautions that one must distinguish between an increased size and weight of the whole gland, and the hypertrophy of only certain tissues. He claims that a disproportion exists in various species between the fibromuscular stroma and glandular epithelium. Hence, if the glandular portion predominates, as in mice, the administration of a substance which arrests secretion will result in diminution in the size of the gland despite an increase in the fibromuscular stroma. On the other hand, the administration of a substance, such as testosterone, which stimulates glandular secretion, evokes an increase in the size of the gland in mice.

2. *Human Bio-Assay*.—Gallagher<sup>11</sup> and his associates have demonstrated marked fluctuations in the daily urinary excretion of the male and female sex

hormones in men and women. Dingemans<sup>12</sup> and his collaborators found the hormones in the urine of both sexes in equal proportions, but noted a decrease of the male sex hormone before puberty and with advanced age, implying that less is secreted. Rusch and Kundest<sup>13</sup> have shown that the excretion of male sex hormone is decreased in proportion to that of estrogenic hormone in men with enlargement of the prostate. The observations of Owen and Cutler<sup>14</sup> are interesting, but they neither add nor detract from the above conclusions. While they found no increase in the urinary prolans nor in the other substances acting like the female sex hormones in the urine of patients suffering from prostatic hypertrophy, as compared to the normal, they failed to report on the quantities of the male sex hormone.

3. *Clinical Observations.*—Von Coppelien<sup>15</sup> treated 50 men with benign hypertrophy of the prostate by intramuscular administration of a crude testicular extract (Homberal) and reported cures in 25 (50 per cent) without diminution in prostatic size. Similar results were observed with pure crystalline male sex hormone by Zuckerman.<sup>9</sup> Lower and McCullagh<sup>16</sup> reported encouraging results in patients with prostatism by oral administration of a crude desiccated extract of beef testes named "inhibin" by them, and alleged to be a definite hormone which inhibited the pituitary gonadotropin. Since their extract included the entire testicular substance, it is difficult to understand how the testosterone fraction was eliminated. Moreover, the observations of Hamilton, Heslin, and Gilbert<sup>17</sup> do not support the theory that improvement of benign prostatic hypertrophy may be obtained by inhibition of the pituitary gland. They treated 9 prostatic males, over a period of twenty-six days, with daily doses of from 500 to 1,500 international units of estrogen (theelin) which has marked inhibitory effects on the pituitary without obtaining any improvement in the urinary disturbance. Walther and Willoughby<sup>18</sup> treated 15 patients with prostatic hypertrophy with intramuscular and oral administration of androstine with excellent results. They, however, also employed prostatic massage.

*The Present Study.*—I was attracted to the problem of prostatic hypertrophy by the acute retention of a man of 60, with jaundice, hepatomegaly, and auricular fibrillation incident to arteriosclerosis, in whom surgery was not advisable. An indwelling catheter was introduced into the bladder for seventy-two hours. Testosterone propionate (Oreton) in doses of 5 mg., three times weekly, was administered intramuscularly. Within three weeks the patient's nycturia was reduced from five or six times to two or three times. Treatment was continued for an additional three weeks with excellent results, nycturia occurring only once or twice. For two weeks following withdrawal of treatment he felt perfectly well and then developed sudden recurrent acute retention, necessitating catheterization. Believing that perhaps larger doses of testosterone propionate would be of more value in prostatism, the present study was undertaken among the inmates of two Jewish Sheltering Homes for the Aged.

This study was purely clinical, the following data being recorded for each patient:

(1) A definite history of prostatism with urinary frequency; difficulty in starting the stream; dribbling; necessity of catheterization or of any surgical treatment of the prostate, and the duration of symptoms.

(2) A general physical examination determining chiefly the state of the cardiovascular system; the presence or absence of hypertension, and the size of the prostate, as determined by digital rectal examination. No laboratory examinations were made.

(3) Results of administration of testosterone propionate.

Twenty-seven patients were observed; 19 in the two above-mentioned institutions; 4 in the wards and clinics at the Mount Sinai Hospital, and 4 in private practice. The youngest was 60 and the oldest was 86. Most of them were hypertensive and arteriosclerotic; a few had residual evidence of a cerebrovascular episode. All were ambulatory and none were decompensated. The duration of the prostatic symptoms varied from 3 to 18 years. The degree of prostatic enlargement, varying clinically from plus 1 to plus 4, had no relation to the clinical manifestations. Some patients with plus 4 enlargement had nocturia four or five times, whereas others with plus 1 enlargement had a nocturnal frequency of from ten to twelve times. Three had undergone prostatectomies twenty, eight, and two years previously, respectively. In only 2 of these was a hard prostate palpable, yet all of them had distressing urinary symptoms. Treatment was limited to testosterone propionate. No patient was catheterized nor were any medications prescribed. Their manner of living was not changed during the course of treatment. The diet and water intake remained the same.

The material employed in this study is "testosterone propionate," a propionic acid ester of testosterone in a solution of oil. Its contents of active hormone are in accordance with the standards of the League of Nations.<sup>19</sup>

*Amount of Testosterone Propionate Administered.*—Testosterone propionate was administered intramuscularly twice weekly. The dose employed was either 5 mg. (15 patients) or 10 mg. (12 patients). Improvement was noted after the administration of from 40 to 50 mg. in 18, and after 60 to 70 mg. in 5 patients. The remaining 4 patients who subsequently received larger doses which totaled from 100 to 110 mg., failed to improve. Once improvement was noted, the dosage was reduced to 10 mg. once weekly. Treatment was continued for three months. Among 8 patients whose treatment was discontinued for 6 weeks, 2 had recurrence of symptoms which were controlled by 3 weekly doses of 10 mg. each.

## RESULTS

Twenty-three patients (85.2 per cent) showed definite clinical improvement and 4 (14.8 per cent) patients failed to respond. The nature of the improvement included the following:

1. A general stimulation which may be attributed either to the increased rest because of the diminished nocturia or to a specific hormone factor.
2. Increase in the force of the urinary flow and greater ease in starting the stream. In 4 patients severe colicky pains, radiating to the urinary meatus during micturition, appeared when the stream became stronger, suggesting the presence of vesical calculi.

3. Dramatic diminution of urinary frequency. The nocturia in 20 of the 23 improved patients was decreased to not more than twice. Three anxiously complained that they had ceased to void even though their daily water intake

amounted to 2 or 3 liters. However, no edema was demonstrable in any of the 3 patients nor did they have excessive perspiration or vomiting, implying that they had unconsciously exaggerated the diminution which, nevertheless, was marked.

4. Dribbling and clothes-wetting completely disappeared in 3 patients, and enuresis was cured in 1.

5. There was no diminution in the size of the prostate in any patient, not even in those patients with marked improvement.

Of the 4 patients who failed to respond to treatment, one probably received insufficient dosage, 2 others had a long history suggestive of calculi with urinary infection, and the remaining one was a markedly neurotic business man. The latter had frequent urination but no residual urine, and his urinalysis was normal. In this instance the frequency was probably psychogenic in origin, despite the presence of prostatic hypertrophy.

*Mechanism of the Response to Testosterone.*—The mechanism by which improvement of prostatism occurs following testosterone treatment may be simply explained on the basis of physiologic alterations rather than on gross structural changes. This opinion is based on the following evidence:

1. There is general agreement that an alteration of either the glandular tissue around the prostatic urethra or the subcervical glands of Motz and Albarran scattered over the trigone of the bladder is the origin of the so-called adenomatous enlargement of the prostate which actually causes the urinary symptoms.

2. Animal experimentation revealing the metaplasia from the normal columnar epithelium of the prostate gland to squamous epithelium under stimulation of estrogen and the reversal to columnar epithelium upon the introduction of androgen is very suggestive that the changes are physiologic in nature. Moreover, the secretion of the epithelial glands is arrested by estrogen and stimulated by androgen. Recalling that androgen is diminished with age, and that according to Champy<sup>20</sup> and his associates, a mucoid edema around the vessels of the verumontanum is normally present in the healthy adult animal but not in aged animals, indicates that histologic changes are the result of physiologic alterations.

3. There is no relationship between the size of the prostate and the clinical symptoms. Conversely, as the clinical symptoms subside the prostatic hypertrophy remains stationary.

4. When recurrence of urinary frequency after cessation of testosterone propionate treatment takes place, symptoms will quickly disappear following the administration of about 30 mg. of the substance, suggesting a rapid physiologic change.

#### COMMENT

Current evidence from animal experimentation probably justifies the conclusion that the unantagonized action of the female sex hormone, estrogen, in aging males is responsible for the prostatic hypertrophy and its subsequent clinical manifestations. The beneficial results obtained by the administration of the male sex hormone, testosterone propionate, are probably caused by local

effects on the glandular portion of the prostate resulting in a reversal of the morbid physiology.

Although the present group of patients is comparatively small, the nearly uniformly favorable results are impressive, especially since no other form of treatment besides testosterone propionate was instituted. While this limited study does not justify definite opinions on proper dosages of testosterone propionate, I am of the opinion that the initial dose should be at least 10 mg. every third day until improvement results, after which time 10 mg. should be given weekly for three to four months. In some instances larger doses may be required. In order to determine the optimal dosages, accurate bio-assay of the urinary excretion levels of the hormone, as applied by Mazer and Israel<sup>21</sup> in the management of estrogen administration, will be necessary.

Urologists frequently consult internists for an opinion concerning the feasibility of surgery in many patients with broken-down cardiovascular systems or with other degenerative changes of old age on whom an operation may mean death. If organotherapy can help these miserable patients before they reach the stage of retention and infection, how much morbidity and mortality could be prevented? Such an accomplishment would not mean the end of surgical treatment, for many cases would still require surgery.

An additional problem of interest is the possibility of overlooking an associated malignancy of the prostate should testosterone prove a real boon. However, not all malignancies of the prostate are diagnosed at present preoperatively, since from 10 per cent to 16 per cent are recognized postoperatively.

#### SUMMARY

1. A review of the current hypothesis concerning the endocrinopathic etiology of benign prostatic hypertrophy is presented.

2. Twenty-seven ambulatory patients with severe prostatism as a result of prostatic hypertrophy were treated intramuscularly with administered testosterone propionate in doses of from 5 to 10 mg. twice weekly for periods of from two to three months.

3. Of the 27 patients 23 (85.2 per cent) were markedly improved, and 4 (14.8 per cent) failed to respond.

I wish to express my appreciation to Doctors Gershon Ginsburg and Harold Nichols, medical directors of the two Jewish Homes for the Aged, for the privilege of studying these patients, and to Dr. Max Gilbert, of the Schering Corporation, for his generosity in supplying the testosterone propionate employed in this study.

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## THE COMPARATIVE IODINE CONTENT OF BLOOD AND CEREBROSPINAL FLUID\*

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THE majority of patients with hyperthyroidism present an elevated blood iodine<sup>1</sup> and an increased loss of iodine in the urine.<sup>2</sup> At present few data<sup>3</sup> concerning the iodine content of the cerebrospinal fluid in hyperthyroidism are available. The presence of iodine in the cerebrospinal fluid has been demonstrated by several investigators.<sup>4-7</sup> The results reported, however, are not consistent. The introduction of Matthews'<sup>8</sup> modification of the Leipert method for the microanalysis of iodine makes possible a more accurate determination of small amounts of iodine. Our purpose in the present communication is to report the results of an investigation of the comparative iodine content of whole blood and cerebrospinal fluid in patients with and without hyperthyroidism.

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## METHODS

Whole blood and cerebrospinal fluid iodine determinations were made on two groups of patients. Ten patients did not have demonstrable thyroid disease, but 2 had toxic nodular goiter and 8 exophthalmic goiter. These patients were afebrile and did not present evidence of central nervous system disease. They had not received iodine in any form for at least one month prior to this investigation. The 10 patients with hyperthyroidism eventually had a thyroidectomy, and the clinical diagnosis was confirmed by the operative and pathologic findings.

The lumbar puncture was made on the morning of the basal metabolism determination. The patients were given orally 3 grains of nembutal for sedation. From 20 to 30 c.c. of spinal fluid was removed, 5 c.c. of which was used for cell and protein study. To prevent possible contamination of the cerebrospinal fluid by the relatively high iodine level of the blood, the first few drops of the fluid after the removal of the wire stylet were discarded. The initial pressure of the fluid was measured and found to be within normal limits in all patients. The fluids did not contain an abnormal number of cells, and the protein contents were normal.

The venepuncture was performed immediately following the lumbar puncture. A sufficient amount of blood was obtained to permit an analysis of 25 c.c. of whole blood and 20 c.c. of serum. The results obtained from the determinations of the iodine content of the whole blood and serum, together with the cell volume, were used as reported<sup>9</sup> to rule out any increased amount of nutritional iodine in the blood. All patients studied revealed a low nutritional iodine indicating that there had been no recent increased intake of iodine.

Analyses of both the whole blood and cerebrospinal fluid iodine were made by one of the authors (R. L. B.), using Matthews'<sup>8</sup> modification of the Leiperl method. This method is accurate to within 5 per cent for amounts of iodine from 2 to 400 micrograms. When a single determination is made upon a specimen containing an amount below 0.5 micrograms of iodine, the result may be in error to the extent of 0.04 microgram. Matthews' method has been used in this clinic since August, 1935, and gives normal blood iodine values of about 4 micrograms per cent.

TABLE I

THE COMPARATIVE IODINE CONTENT OF WHOLE BLOOD AND CEROBROSPINAL FLUID IN PATIENTS WITHOUT THYROID DISEASE

CASE NO.	DIAGNOSIS	SEX	AGE	B.M.R.	BLOOD IODINE		SPINAL FLUID IODINE
					%	µg%	µg%
1	Exostosis of femur	M	18	Minus 5		2.9	0.4
2	Obesity	F	36	Plus 4		3.1	0.8
3	Psychoneurosis	M	30	Plus 6		3.2	0.6
4	Normal	F	25	Minus 3		3.3	0.8
5	Osteoarthritis of knee	M	38	----		3.4	0.4
6	Ovarian cyst	F	24	----		3.4	0.5
7	Fracture of radius	M	22	Plus 17		3.4	0.4
8	Ulcer of leg	M	40	----		3.9	0.8
9	Psychoneurosis	F	26	Plus 6		4.1	0.6
10	Atrophic arthritis	M	30	Plus 12		5.1	0.2
Average						3.6	0.5

## RESULTS

The ages of the 10 patients without evidence of thyroid disease ranged from 18 to 40 years. The basal metabolic rates were within normal limits in seven determinations. The blood iodine ranged from 2.9 to 5.1 micrograms per cent, with an average of 3.6. This is at the lower range of normal for patients in this region using Matthews' method of analysis. The cerebrospinal fluid iodine ranged in values from 0.2 to 0.8 microgram per cent, with an average of 0.5 microgram per cent. The results obtained are presented in Table I.

There was an elevation of the basal metabolic rate in all the patients with hyperthyroidism (Table II). It ranged from plus 23 to plus 84, with an aver-

TABLE II

THE COMPARATIVE IODINE CONTENT OF WHOLE BLOOD AND CEREBROSPINAL FLUID IN PATIENTS WITH HYPERTHYROIDISM

CASE NO.	DIAGNOSIS	DURATION OF SYMPTOMS	SEX	AGE	B. M. R.	BLOOD IODINE	SPINAL FLUID IODINE
					%	μg%	μg%
1	Toxic nodular goiter	10 years	F	28	Plus 26	5.1	0.4
2	Exophthalmic goiter	2 years	F	38	Plus 40	5.5	0.5
3	Exophthalmic goiter	1 year	F	61	Plus 56	6.1	0.2
4	Toxic nodular goiter	20 years	F	45	Plus 36	6.7	0.6
5	Exophthalmic goiter	2 years	F	38	Plus 23	8.8	0.8
6	Exophthalmic goiter	1 year	F	36	Plus 63	8.9	0.4
7	Exophthalmic goiter	1 year	M	24	Plus 84	9.6	0.4
8	Exophthalmic goiter	6 months	F	40	Plus 39	10.8	0.5
9	Exophthalmic goiter	6 months	M	59	Plus 65	11.2	0.3
10	Exophthalmic goiter	6 months	F	43	Plus 64	15.7	0.8
Average					Plus 50	8.8	0.5

age of plus 50 per cent. Their ages ranged from 24 to 63 years. The blood iodine was increased in all 10 patients. This increase was most marked in those with a more recent onset of the disease. The lowest value of 5.1 micrograms per cent was found in a patient with toxic nodular goiter of ten years' duration. In a woman with exophthalmic goiter of 6 months' duration and a basal metabolic rate of plus 64, the blood iodine was 15.7 micrograms per cent, which is four times normal. The average increase was a little over twice the normal as found in this region and as compared with the average blood iodine of the 10 patients without thyroid disease. The spinal fluid iodine concentration was similar to that found in the individuals without thyroid disease, ranging from 0.2 to 0.8, with an average of 0.5 microgram per cent.

## LITERATURE

Methods for the determination of the iodine content of organic materials, prior to the introduction of Kendall's procedure<sup>10</sup> in 1920, were inadequate to demonstrate consistently the presence of iodine in the normal spinal fluid. In 1921 Osborne<sup>4</sup> published his analyses of pooled spinal fluid obtained from patients not receiving iodine. He used Kendall's method. The average of nine determinations was found to be 18 gamma\* per cent. Following the administration of iodides there ensued a rise in the cerebrospinal fluid iodine, this being most marked in patients with meningitis.

\*Gamma and microgram are synonymous, designating 0.001 mg.

Cohen<sup>11</sup> in 1924 was unable to demonstrate the presence of iodine in the spinal fluid of 7 patients without meningeal involvement, each of whom had received 50 c.c. of 10 per cent potassium iodide solution intravenously from twelve to sixty minutes previous to the lumbar puncture. In 3 patients with meningeal disease his starch iodine test was positive.

Campbell and Snodgrass,<sup>12</sup> using Kendall's method, found only a trace of iodine in the spinal fluid of 19 patients with and without meningeal involvement, who had not received iodine for several months previously.

Hirsch<sup>3</sup> in 1930 determined both the blood and spinal fluid iodine in normal persons and in patients with Basedow's disease. With an average blood iodine of 13 gamma per cent the normal spinal fluid iodine was found to be 10 gamma per cent. In Basedow's disease the iodine in the cerebrospinal fluid was 70 to 90 per cent of the blood iodine as compared to a percentage of 65 in normal persons.

Hahn and Schürmeyer<sup>5</sup> used von Fellenberg's method for the determination of iodine in the cerebrospinal fluid of 17 patients who had not previously received iodine. Of these patients 12 had syphilis of the central nervous system. With the blood iodine level at 10.6 gamma per cent the average concentration of iodine in the cerebrospinal fluid was found to be 7.4 gamma per cent. Following the administration of iodides the cerebrospinal fluid iodine concentration was increased. This was augmented by artificially induced fever.

In 1935 Castex and Schteingart<sup>6</sup> determined the iodine content of the cerebrospinal fluid obtained from 25 patients, using the method of Leitch and Henderson. In 4 patients 2 to 2.5 gamma per cent was found, while in the remainder only traces of iodine could be demonstrated. Some of the patients had abnormal cerebrospinal fluid, but no relation of the iodine level to any other alterations was found.

McCullagh<sup>7</sup> analyzed the blood and spinal fluid of 11 patients who were undergoing encephalographic studies and who, except in one instance, had no thyroid disease. He used his own method of microanalysis. With a blood iodine averaging 7.7 gamma per cent, the spinal fluid iodine was 2.0 gamma per cent.

#### DISCUSSION

Our findings thus confirm the reports of previous investigators that iodine is a normal constituent of the cerebrospinal fluid. However, our iodine values for both the whole blood and cerebrospinal fluid are definitely lower than those of earlier investigators. Moreover, the spinal fluid iodine was 14 per cent of the blood iodine in patients without thyroid disease, and only 6 per cent in those with hyperthyroidism.

The amount of cerebrospinal fluid analyzed was 15 to 25 c.c. This contains from 0.075 to 0.125 microgram of iodine. The reagent blanks for these analyses were 0.06 microgram. An error of approximately 15 per cent in the analysis of cerebrospinal fluid must consequently be considered.

Osborne<sup>4</sup> was the first to suggest an increased permeability of the choroid plexus to iodides in meningeal diseases. Hahn and Schürmeyer<sup>5</sup> found that fever increased this permeability. In our investigation we were particularly careful to rule out all patients with meningeal involvement, those with fever, and

also those who had received iodine recently. Each of these three factors might result in an abnormal increase in the cerebrospinal fluid iodine.

Nothing is known of the true nature of the cerebrospinal fluid iodine. It may possibly be in a diffusible form, similar to the iodine found in the red blood cells.<sup>9</sup> However, there was no increase in the cerebrospinal fluid iodine in the patients with hyperthyroidism, even though there was an increase in the blood iodine. This suggests that the thyroid hormone does not circulate in the cerebrospinal fluid. If the thyroid hormone were normally present in cerebrospinal fluid, one would expect an increase in the iodine content in hyperthyroidism similar to the increased blood iodine.

#### SUMMARY AND CONCLUSION

Ten patients without thyroid disease, fever, or meningeal disease, who had been on a relatively low iodine intake for at least one month, presented an average blood iodine of 3.6 micrograms and a cerebrospinal fluid iodine of 0.5 microgram per cent.

Ten patients with hyperthyroidism, who had not received iodine for one month, had an average blood iodine of 8.8 micrograms and a cerebrospinal fluid iodine of 0.5 microgram per cent.

The average cerebrospinal fluid iodine was the same in the patients both with and without hyperthyroidism, even though there was an average increase of 144 per cent in the blood iodine in the patients with hyperthyroidism as compared to the patients without thyroid disease.

Iodine is a normal constituent of the cerebrospinal fluid. There is no increase in the cerebrospinal fluid iodine in patients with hyperthyroidism.

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## INTUBATION STUDIES OF THE HUMAN SMALL INTESTINE\*

### XVI. THE BACTERIAL FLORA OF THE ILEUM COMPARED WITH THAT OF THROAT AND STOMACH IN NORMAL SUBJECTS

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THE development by Miller and Abbott<sup>1</sup> of a satisfactory technique for rapid intubation of the small intestine of man has made it possible to undertake a study of the bacterial flora of that organ and to compare the observations with those on specimens, collected almost simultaneously, from the upper and more accessible portions of the alimentary tract. By a slight variation in the original two-lumened apparatus, uncontaminated specimens from the ileum may usually be obtained within a few hours. The collection of suitable specimens from several areas in the normal affords an opportunity to study the fate of the usual organisms as they pass along the tract, and therefore to establish standards for subsequent investigation of bacterial flora under abnormal conditions.

#### REVIEW OF LITERATURE

Torrey<sup>2</sup> observed in dogs that *B. coli* were numerous in the ileum, that *B. welchii* were even more common, and that putrefaction occurred when inoculations were made on a sugar-free medium. Arnold,<sup>3</sup> working on dogs with non-leaking fistulas, observed in the ileum a very rich bacterial flora with a predominance of coliform types. Daek and Petran<sup>4</sup> studied the bacterial activity in different levels of the intestine of the dog and monkey by means of non-leaking fistulas and found the green streptococcus usually present in the ileac contents.

Bacterial studies on the human small intestine, particularly the lower portion, seem to have begun with van der Reis<sup>5</sup> who used, at first, an automatic cartridge, which could be opened at the desired level by an electric magnet, and later an Einhorn tube. He concluded that for man there was an obligate flora of the small intestine. In fasting normal subjects he found gram-negative bacteria predominating in the ileum. Four groups were present: (a) *Streptococcus lacticus*, (b) *Bacillus lactis aerogenes*, (c) *Bacillus lactis*, and (d) *Bacillus coli communis*. Goldman,<sup>6</sup> using a duodenal tube with a gelatin capsule over the bucket, made some tests on material, chiefly from the jejunal level, under normal conditions of digestion. She reported on 4 normal subjects and 20 hospital cases. In the normal persons she found that the viable count varied within wide limits. Spore-bearing aerobes were present in all cases. Anaerobes

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were not present. Gram-negative bacilli of the colon group, gamma streptococcus and *B. acidophilus* appeared to be normal features. Bogendörfer<sup>7</sup> concluded from a study of 15 normal subjects under fasting conditions that the small intestine possessed a specific, but not particularly varied, flora of which the *B. coli* was not normally present. Thomson, Einhorn, and Coleman<sup>8</sup> used a collodion membrane over the end of a special rubber tube to obtain intestinal samples. They studied the predominant organisms of the throat, stomach, duodenum, and small intestine in 2 normal subjects and 10 patients. In the normal subjects streptococci and spirilla predominated in the throat, streptococci in the stomach, and a gram-negative colonlike organism in the jejunum and ileum.

#### SUBJECT AND TECHNIQUE FOR SECURING SPECIMENS

The present report is based on a comparative study of the organisms isolated under fasting conditions from the throat, stomach, and ileum in 8 apparently normal adults who had no upper respiratory or gastrointestinal disease. Interest was centered on the flora of the ileum since that of the duodenum and its dependence on the physiologic status of the stomach are known.

1. For the throat specimens, after gargling with a normal saline solution, the base of the tongue and the posterior pharyngeal wall were rubbed with a sterile swab.

2. The stomach specimens were taken immediately afterwards by means of a sterilized duodenal tube with a Rehfuß tip, the latter enclosed in a thin rubber sheath. After the tube had been in the stomach for ten minutes, the rubber over the tip was broken by blowing air into the tube from a sterile syringe, and then the stomach specimen was aspirated in the usual fashion into a sterile container.

3. For the collection of ileac specimens, a sterile Miller-Abbott<sup>1, 9</sup> double-lumen tube with a covered tip was introduced immediately after removing the gastric tube. The intubation, which required one to four hours, was accomplished with the aid of a fluoroscope. The bag attached distally to one lumen of the tube and distended during the intubation was deflated when the desired area of the bowel was reached to allow free passage of intestinal contents. The rubber sheath over the perforations into the other lumen was then ruptured, thus for the first time permitting intestinal material to enter the tube, and ileac fluid was withdrawn.

#### PROCEDURE

The studies were commenced as soon as the specimens were collected. Hydrogen-ion determinations were made by the Meeker and Reinhold quinhydrone electrode method. Direct smears of the stomach and ileac contents were stained by Gram's method.

Plate and hemocytometer counts were made of the ileac contents. For the plate count, dilutions of 1:200 and 1:20,000 were made, and 0.5 c.c. of each dilution was plated in duplicates on plain agar and incubated aerobically and anaerobically for four days, after which time counts were made. Direct counts were made by hemocytometer, the contents being diluted 1:20, using Löffler's methylene blue as a stain. A Levy counting chamber was employed.

Throat cultures were made on plain and blood (human) agar in duplicates, aerobic and anaerobic. Beef infusion broth, pH 7.2, was the basis of all culture media.

Stomach and ileac contents were made on human blood, chocolate, Torrey, and Sabouraud media, and on bromthymol blue lactose agar, plain and bicarbonate, brain glucose broth, deep meat broth, and deep milk. Blood and chocolate plates were made in duplicates, aerobic and anaerobic, with exception of one chocolate plate under carbon dioxide. Deep meat and deep litmus milk were inoculated with 0.1 c.c. of contents heated to 80° C. for ten minutes.

Anaerobic cultures were studied by means of the improved McIntosh and Feldes anaerobic jar. The air in the jar was exhausted by means of a vacuum pump, and then hydrogen gas was permitted to flow slowly into the jar. Anaerobiosis was checked with methylene blue and a tube of *B. welchii*. Determinations were made by standard methods. Organisms were classified according to Bergey and the Committee of the Society of American Bacteriologists (1930).

#### OBSERVATIONS

The hydrogen-ion concentration of the stomach contents of the normal adult, as shown in Table I, varied from 1.74 to 7.01. The acid-base factor showed an influence on the bacterial flora of the stomach contents. The specimens with a pH ranging from 1.74 to 2.48 and with free hydrochloric acid gave no bacterial growth on any medium. One with a pH of 4.2 and free acid showed only a sarcina and a mold (*syringospora*). One with a pH of 4.79 and no free acid showed abundant bacterial growth. One with a pH of 5.08 and no free acid showed no growth in any medium incubated aerobically but under anaerobic conditions showed moderate growth; three different groups were identified, one of which was an obligate anaerobe. In general, stomach contents with a pH above 4.2 showed moderate to abundant bacterial growth.

The ileac fluid of the normal adult was yellowish to greenish brown in color, viscous, and passed with difficulty through a Berkefeld filter. Its pH varied from 6.59 to 7.60. All cultures of the ileum were positive. More colonies grew when the ileac fluid was alkaline. Fewer organisms and fewer types grew when it was acid.

The direct examination of the stomach contents by the Gram method showed gram-negative and gram-positive cocci in pairs and short chains as well as gram-negative and gram-positive bacilli. There were moderately few microscopically visible bacteria in those stomach contents which gave no growth. Gram-positive types were numerous in those specimens in which the cultures were positive.

The direct examination by Gram's method of the ileac contents showed a predominance of gram-negative bacteria in all specimens.

The bacterial count of the ileac contents by plate and direct count showed marked variation (Table I). The plate count, that is, the count of viable bacteria, averaged from 144,000 to 3,600,000 per c.c. under aerobic incubation and from 166,000 to 3,240,000 per c.c. under anaerobic incubation. There was no constant relationship between the aerobic and anaerobic yields, but the latter was usually higher.

TABLE I

STOMACH			ILEUM			
CASE NUMBER	FREE HCl	pH	pH	BACTERIAL COUNT NO. PER C.C.		
				PLATE METHOD		HEMO-CYTOMETER METHOD
				AEROBIC CULTURE	ANAEROBIC CULTURE	
100	Not made	Not made	Not made	Not made	Not made	Not made
103	None	7.22	7.56	3,600,000	3,240,000	216,000,000
104	None	7.01	7.36	900,000	1,002,000	64,000,000
106	+	1.74	6.59	144,000	160,000	38,000,000
107	None	4.79	6.68	1,004,000	2,886,000	Not made
109	+	2.48	7.12	948,000	996,000	56,000,000
110	+	4.2*	7.3*	516,000	672,000	67,000,000
113	None	5.08	7.60	Not sufficient	Not sufficient	120,000,000

\*Universal Indicator.

The direct count varied from 38,000,000 to 216,000,000 per c.c. With the exception of one case, the direct count averaged seventy times as high as the plate count. This variation may be due to the fact that many organisms were dead or that they did not grow well on infusion agar.

## BACTERIAL VARIETIES AND DISTRIBUTION

In the bacterial study 435 colonies were picked from plates, and of this number 331 were tested biochemically, the remainder being discarded because of similarity.

From the tabulation in Table II the different groups and species of bacteria and the frequency with which they occurred in the throat, stomach, and ileum of the normal adult may readily be seen. Table III shows groups and species of bacteria according to location in each of the cases.

In the throat there were identified 14 groups and 31 species of bacteria and 1 group each of molds and yeast.

In the stomach contents there were identified 14 groups with 27 species of bacteria and 1 group each of molds and yeast.

In the ileum there were identified 18 groups and 42 species of bacteria and 1 group each of the molds and yeast.

In the throat the *Streptococcus* group was identified in all cases. The next most frequent group was *Neisseria*, isolated in 5 of the 8 cases. In relatively small numbers were *Staphylococcus* of the hemolytic type, *Micrococcus* (nonhemolytic) and *Sarcina*, and occasionally the pneumococcus, *Klebsiella*, *Fusiformis*, *Actinomyces*, spore-bearing aerobic bacillus, *Alcaligenes*, *Bacteroides*, and *Syringospora*.

In the stomach contents the groups most frequently present were *Streptococcus* and *Micrococcus* (nonhemolytic). These were present in 3 of the 7 cases studied. The next most frequent groups were *Neisseria*, *Staphylococcus* (hemolytic type), and *Pseudomonas*. Occasionally identified were *Escherichia* of an unusual variety, pneumococcus, *Alcaligenes*, *Actinomyces*, *Bacteroides*, and *Syringospora*.

The group of bacteria most frequently identified in the ileac contents was *Streptococcus*, not unlike those found in the respiratory tract. The next most frequent groups were *Micrococcus* (nonhemolytic) in 7, *Staphylococcus* (hemo-



TABLE II

NUMBER OF TIMES GROUPS AND SPECIES OF BACTERIA AND MOLDS WERE IDENTIFIED IN 5  
THROAT AND ILEAC CONTENTS AND 7 STOMACH CONTENTS

GROUPS	SPECIES	THROAT	STOMACH	ILEUM
Pneumococcus		1	2	2
	type III	1	1	1
	type sp?	0	1	1
Streptococcus		8	3	8
	alpha	8	3	8
	beta	3	1	4
	gamma	4	2	5
Neisseria		5	2	2
	flavus	0	0	1
	perflavus	2	0	0
	catarrhalis	3	2	1
Staphylococcus		3	1	6
	albus	2	1	4
	aureus	1	0	3
	epidermis	0	0	2
Micrococcus		3	3	7
	varians	0	1	1
	flavus	1	1	0
	subnitens	0	1	0
	sphaeroides	0	1	2
	candicans	1	0	2
	candidus	2	2	2
	luteus	1	0	0
	caseolyticus	0	1	0
	luteolus	0	0	1
	freudenreichii	0	1	0
	sp?	3	0	2
Sarcina		3	2	5
	lutea	1	0	1
	psychrocarterica	1	1	1
	subflava	0	0	1
	flava	0	1	0
	ventriculi	0	0	1
	sp?	3	0	2
Vibrio		0	0	1
	sp?	0	0	1
Pseudomonas		0	2	2
	myxogenes	0	1	1
	centrifugans	0	1	1
Achromobacter		0	1	0
	sp?	0	1	0
Lactobacillus		0	0	2
	acidophilus A	0	0	1
	acidophilus B	0	0	1
Klebsiella		1	0	1
	capsulatus	1	0	1
Escherichia		0	1	2
	enterica	0	0	1
	neapolitana	0	1	1
Shigella		0	0	3
	minutissima	0	0	1
	sp?	0	0	1

TABLE II—CONT'D

GROUPS	SPECIES	THROAT	STOMACH	ILEUM
Alcaligenes		1	1	1
	metacaligenes	1	0	0
	recti	0	1	1
Bacteroides		1	1	1
	fragilis	0	1	0
	sp?	1	0	1
Bacillus aerobic spore-bearing		1	1	3
	adhaerens	0	0	2
	circulans	0	0	1
	flexus	1	1	0
Actinomyces		2	2	4
	actinomorphus	2	0	0
	erythropilis	1	1	3
	sp?	0	1	1
Corynebacterium		3	2	3
	xerosis	1	1	1
	pseudodiphtheriae	1	1	1
	lymphophilum	0	1	0
	enzymicum	0	0	2
	flavidum	1	0	0
	sp?	0	0	1
Fusiformis		1	0	0
	dentium	1	0	0
Saccharomycetaceae		1	0	3
	sp?	1	0	3
Syringospora		1	1	1
	psilosis	1	1	1
Group? gram-positive rod		1	0	0

lytic) in 5, and *Sarcina* in 5 of the 8 cases. The other groups present in relatively small percentage were spore-bearing aerobic bacilli, *Corynebacterium*, pneumococcus (which was isolated only when the stomach contents had no free acid and a slightly alkaline reaction), *Neisseria*, *Pseudomonas*, *Lactobacillus*, *Shigella*, *Actinomyces*, and *Escherichia*. Present in scattered numbers were *Klebsiella*, *Alcaligenes*, *Bacteroides*, *Vibrio*, *Saccharomycetaceae*, and *Syringospora*.

Table III shows the predominating organisms as determined from directly inoculated aerobic and anaerobic blood agar plates. In the throat, no single predominating organism was noted in 8 patients unless it was streptococcus. *Streptococcus alpha* was the predominating organism in the stomach and ileac contents.

Work has been begun on a comparative study of the bacteria in the throat, stomach, and ileum of the abnormal person by the Miller-Abbott intubation method. The investigation has been completed in 4 patients; 2 with ulcerative colitis, one with hypertension with septic sore throat, and one with an intestinal fistula. It suggests that bacterial growth is less extensive than under normal conditions in that fewer kinds of bacteria are present.

#### COMPARISON OF GROUPS IN THROAT, STOMACH, AND ILEUM OF NORMAL ADULTS

Streptococci were identified in all throat and ileac specimens and in the stomach contents of 3 patients. Of the types isolated alpha was present in all

TABLE III  
BACTERIAL FLORA ACCORDING TO CASE AND AREA\*

CASE NO.	THROAT	STOMACH	ILEUM
100	Streptococcus alpha Staphylococcus albus*†  Micrococcus sp? Klebsiella capsulatus	Not studied	Streptococcus alpha Streptococcus beta Neisseria catarrhalis Staphylococcus albus Micrococcus varians Micrococcus spheroides Sarcina lutea Klebsiella capsulatus*† Bacillus adhaerens Bacillus circulans Actinomyces sp? Saccharomycetaceae sp†
103	Pneumococcus type III Streptococcus alpha*† Streptococcus beta Streptococcus gamma Neisseria perflavus Sarcina lutea Corynebacterium xerosis	Pneumococcus type III Streptococcus alpha*† Streptococcus beta Micrococcus varians Micrococcus flavus Micrococcus subcitreus Micrococcus spheroides Alcaligenes recti	Pneumococcus type III Streptococcus alpha*† Streptococcus beta Micrococcus candicans Sarcina psychrocarctica Alcaligenes recti Actinomyces erythropolis Corynebacterium enzymicum
104	Streptococcus alpha Streptococcus beta Streptococcus gamma* Neisseria catarrhalis  Staphylococcus albus Corynebacterium pseudo-diphtheriae†	Pneumococcus type?  Streptococcus alpha Streptococcus gamma*† Staphylococcus albus Neisseria catarrhalis Staphylococcus albus  Micrococcus candidus  Pseudomonas myxogenes Actinomyces erythropolis Corynebacterium pseudo-diphtheria	Pneumococcus type?  Streptococcus alpha Streptococcus gamma* Staphylococcus albus Neisseria flavus Micrococcus candicans†  Micrococcus spheroides Vibrio sp? Sarcina sp? Pseudomonas myxogenes Bacillus adhaerens  Corynebacterium enzymicum Corynebacterium sp†
106	Streptococcus alpha Streptococcus beta Alcaligenes metalcaligenes Corynebacterium flavidum* Bacteroides sp?	All cultures negative	Streptococcus alpha*† Streptococcus gamma Lactobacillus acidophilus A Lactobacillus acidophilus B Shigella minutissima Shigella sp? Actinomyces erythropolis
107	Streptococcus alpha Neisseria catarrhalis* Micrococcus candicans Micrococcus sp?	Streptococcus alpha*† Streptococcus gamma Neisseria catarrhalis Micrococcus candidus Micrococcus caseolyticus Micrococcus freudenreichii Sarcina psychrocarctica Sarcina flava Pseudomonas centrifugans  Corynebacterium xerosis	Streptococcus alpha Streptococcus beta Streptococcus gamma Staphylococcus aureus  Micrococcus sp? Sarcina psychrocarctica Sarcina ventriculi Sarcina sp?*† Pseudomonas centrifugans Bacteroides sp? Vibrio sp? Actinomyces erythropolis Saccharomycetaceae sp†

\*Predominating organism—aerobic plate.

†Predominating organism—anaerobic plate.

‡No predominant group present.

TABLE III--CONT'D

CASE NO.	THROAT	STOMACH	ILEUM
109	<i>Streptococcus alpha</i> <i>Streptococcus beta</i> <i>Streptococcus gamma</i> <i>Neisseria catarrhalis</i> *†  <i>Actinomyces actinomorphus</i> <i>Actinomyces erythropilis</i> <i>Saccharomycetaceae</i> sp?	All cultures negative	<i>Streptococcus alpha</i> *† <i>Streptococcus gamma</i> <i>Neisseria catarrhalis</i> <i>Staphylococcus albus</i>  <i>Staphylococcus aureus</i> <i>Staphylococcus epidermis</i> <i>Micrococcus candidus</i> <i>Sarcina psychrocarterica</i> <i>Saccharomycetaceae</i> sp?
110	<i>Streptococcus alpha</i>  <i>Streptococcus gamma</i> † <i>Neisseria catarrhalis</i> <i>Staphylococcus aureus</i> <i>Sarcina psychrocarterica</i> * <i>Syringospore psilosis</i>	<i>Sarcina flava</i> †  <i>Syringospore psilosis</i>	<i>Streptococcus alpha</i> <i>Streptococcus beta</i> <i>Streptococcus gamma</i> *† <i>Staphylococcus aureus</i> <i>Micrococcus</i> sp? <i>Sarcina psychrocarterica</i> <i>Escherichia enterica</i> <i>Corynebacterium xerosis</i> <i>Corynebacterium pseudo diphtheria</i> <i>Syringospore psilosis</i>
113	<i>Streptococcus alpha</i> † <i>Neisseria perflavus</i> <i>Micrococcus flavus</i> <i>Micrococcus</i> sp?* <i>Sarcina</i> sp? <i>Bacillus flexus</i> <i>Fusiformis dentium</i> <i>Gram-positive bacillus</i> group?	<i>Escherichia neopolitana</i> † <i>Bacteroides fragilis</i> <i>Actinomyces</i> sp? <i>Bacillus flexus</i>	<i>Streptococcus alpha</i> *† <i>Staphylococcus albus</i> <i>Staphylococcus epidermis</i> <i>Micrococcus candidus</i> <i>Escherichia neopolitana</i>

throat and ileac specimens and in 2 from the stomach; the beta type in 5 of the throat, 1 of the stomach, and 3 of the ileac specimens; and the gamma type in 4 of the throat, 2 of the stomach, and 5 of the ileac specimens.

It is of interest that the majority of the beta streptococci, 2 from the throat and 2 from the ileac contents, were isolated only under anaerobic conditions. They were not, however, obligate anaerobes, though all were markedly micro-aerophilic. All produced soluble hemolysins. All retained their ability to give beta hemolysis when grown aerobically on human blood agar.<sup>10</sup>

Later in the study, identification of the beta streptococci was made by Lancefield's standards, using Brown's<sup>11</sup> method for precipitation. Of the 5 throat strains, one was group A, one was group B, the others were not done. The one strain from the stomach contents did not classify by any sera available, A to E, but biochemically was group G. One strain from the ileac contents was group B, while two strains which gave negative precipitin reactions with all antiserum used were biochemically group G.

Hemolytic staphylococcus was twice as frequently identified in the ileac contents as in the throat. It was present in only one stomach specimen. *Staphylococcus albus* was the most frequent species identified.

*Neisseria* were relatively few in the stomach and ileac contents, although they were present in over half the throats studied.

Micrococci which were predominately nonhemolytic were identified in 7 of the 8 ileac specimens. Six different species were noted. They occurred relatively less in the throat and stomach contents, namely, 3 out of 8 cases for the former and 3 out of 7 cases for the latter.

Sarcina were identified in 5 of the 8 ileac contents, 3 of the 8 throats, and only 1 of the 7 stomach contents.

The *Escherichia* group was not observed in the throat. It was identified in 2 of the 8 ileac specimens, and once from the stomach. In the stomach it was isolated from an anaerobic plate which showed a scant flora, the aerobic cultures being sterile.

No *B. coli commune* or *communior* was identified in the three regions studied. The one species of *Escherichia* present in the stomach and two species present in the ileac contents were of uncommon varieties.

No *B. welchii* have been observed in any of the cases to date, despite careful search. Specimens have been inoculated into deep meat and milk after heating to 80° C. for ten minutes and incubated anaerobically. We have readily identified this organism in material obtained from the ulcerative lesion of ulcerative colitis cases and know it to be present sometimes in the presumably normal small intestine.

Three groups of bacteria and one yeast, namely, *Shigella*, *Lactobacillus*, *Vibrio*, and a *Saccharomycetaceae* were observed only in the ileac contents.

A gram-positive, nonacid-fast, nonhemolytic, nonmotile branching organism, belonging to the *Actinomyces* group, was identified in 2 of the 8 throats, 2 of the 7 stomach contents, and 5 of the 8 ileac contents. The microorganism was occasionally observed in direct examination of the ileac contents. Without exception, the organism was isolated upon human blood agar anaerobic plates. Three species were observed according to their cultural and biochemical reaction. All species were microaerophilic. Filament forms were not dominant in primary cultures and frequently not until several subcultures were made. Involution forms on the end of filaments, both clubbed and spherical shaped, were present in cultures incubated for several days.

One species corresponded to *Actinomyces actinomorphus* of Bergey's classification. It gave a luxuriant, white, confluent growth on 0.1 per cent glucose agar, adherent to the medium and difficult to emulsify. It produced no change in milk, liquefied in gelatin, reduced nitrates, produced no indol, formed acid but no gas in sucrose, maltose, levulose, dextrin, and inulin.

Another species corresponded to *Actinomyces erythropolis* of Bergey's classification. It showed a smooth, opaque, nonadherent, white growth on 0.1 per cent glucose agar. It formed acid in milk, did not liquefy gelatin, reduced nitrates in less than twenty-four hours, produced no indol, formed acid but no gas, in glucose, sucrose, lactose, maltose, galactose, salicin, levulose, dextrin, and occasionally in mannite and inulin.

Another species growing on 0.1 per cent glucose agar, like the latter, was inactive in all media except media for reducing nitrates.

The predominating organisms present in the ileac contents of the normal fasting adult were those capable of utilizing sugars, but not of producing indol or liquefying gelatin.

Those organisms capable of digesting proteins, other than the *Staphylococcus* group, were relatively infrequent in the ileac contents. Six groups and 7 species of organisms capable of liquefying gelatin, and 3 groups and 4 species capable of producing indol were identified. In 1 of the 8 cases, no protein-digesting or indol-producing organisms were identified. In 2 cases, gelatin-liquefying types alone were observed, and in 5 cases, both gelatin liquefiers and indol producers were present. It is, of course, known that many organisms will first utilize for energy any carbohydrates present, sparing the proteins.

#### SUMMARY AND CONCLUSIONS

This study of the bacterial flora of the human throat, stomach, and ileum, employing for the first time the Miller-Abbott tube to secure the ileac specimens, indicates, by both aerobic and anaerobic cultures, a marked similarity in the organisms from the three areas. Variation in frequency rather than types of bacteria is noted. The varieties and place of discovery may be seen by consulting Tables II and III.

In the throat, *Streptococcus* and *Neisseria* were the most frequent groups identified.

In the stomach, *Streptococcus* and *Micrococcus* (nonhemolytic) were the most frequent groups.

Ileac fluid showed *Streptococcus* and *Micrococcus* (nonhemolytic) as the most frequent groups.

*Streptococcus alpha* was the predominant group in the stomach and ileac contents.

Certain types of bacteria, such as *B. welchii*, terminal spore-bearing anaerobes, and *B. coli communis* and *communior*, were not observed in the normal fasting adult ileac contents. The *Escherichia* species identified were uncommon varieties.

Direct examination of the contents of the ileum by Gram's method showed gram-negative types of bacteria to predominate.

Biochemical tests showed protein-digesting organisms to be relatively low and the carbohydrolitic organisms to be correspondingly high.

The number of viable organisms growing in laboratory media and the organisms enumerated by hemocytometer count showed a relationship of 1 to 70.

Because certain organisms were microaerophilic, anaerobic methods were necessary for this study. This was especially true for the isolation of the *Streptococcus* and *Actinomyces* group in the throat and ileum. Relatively few obligate anaerobes were identified.

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## LABORATORY METHODS

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### A NEW INSTRUMENT FOR THE DIFFRACTOMETRIC MEASUREMENT OF THE DIAMETER OF RED BLOOD CELLS\*

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THOMAS YOUNG<sup>1</sup> in 1813 first applied the principle of diffraction of light to the measurement of small objects. He made an instrument which he designated an eriometer since it was designed primarily for measuring threads of wool. With this apparatus, he determined the diameter of other small objects such as vegetable spores and red blood cells. The figure he obtained for the mean diameter of human erythrocytes was 6.8 microns. This is not far from the correct measurement.

The principle utilized by Thomas Young is simple. When a beam of white light strikes a small obstacle or is passed through a small aperture, the light waves are "bent" at the sides of the object or aperture. Since the wave lengths of the constituent colors of white light are all different, a spectrum is produced. This is due to the fact that the degree of "bending" is dependent on the wave length of the color. The usual diffraction grating utilized for producing a spectrum consists of many parallel opaque lines closely cut with a diamond point on a glass plate. This produces a spectrum in the shape of a band. If white light is passed through a film of blood cells or other small round objects, a corona is formed of the spectral colors by the same mechanism. The blood film becomes a diffraction grating. The coronas formed by erythrocytes are the composite of tens of thousands of individual cell coronas.

In 1918 Pijper<sup>2</sup> independently utilized the diffraction principle for measuring the diameter of small objects. His instrument was first used for measuring bacteria. In 1919<sup>3</sup> he applied this principle to the measurement of the diameter of erythrocytes. Pijper has made several types of apparatus for the diffractometric measurement of diameter. Zeiss now supplies a practical clinical instrument, the "blood cell tester," for determining the diameter of erythrocytes, made according to Pijper's specifications<sup>4</sup>.

Unaware of Thomas Young's efforts, Pijper independently rediscovered the principles utilized in the diffractometric measurement of small objects. He deserves the greatest credit for the rediscovery, for priority in developing a practical clinical instrument, and for first emphasizing in 1924 the value of such an instrument in the diagnosis of pernicious anemia<sup>5</sup>.

Since Pijper's pioneer work, others have made instruments for measuring the diameter of red blood cells by diffraction. Emmons<sup>6</sup> in 1927 described his

\*From the Cleveland Clinic, Cleveland.

The Haden-Hausser erythrocytometer is manufactured by C. A. Hausser & Son and sold by the Arthur H. Thomas Co., West Washington Square, Philadelphia, Pa.



erimeter; Eve<sup>7</sup> in 1929 the halometer, and Bock<sup>8</sup> in 1933, the erythrocytometer known by his name. The principle of all these devices depends on the fact that the size of the spectrum varies with the size of the red blood cell and its distance from the light source. The diameter of the coronas varies inversely as the size of the particles and also inversely as their distance from the light. If the size of the coronas is kept constant, then the size of the particles will vary directly as their distance from the light source and vice versa. With Pijper's blood cell tester, the distance of the blood film from the light is kept constant and the diameter of the coronas is measured; with the other instruments, the size of the coronas is kept constant in various ways and the distance from the light source is determined.

I have compared measurements of the red cell diameter with the eyepiece micrometer by direct measurement of the cells projected on a screen and with the Pijper, Emmons, and Bock instruments<sup>9</sup>. The Emmons erimeter was found to be practical and to give accurate results. Pijper's blood cell tester is designed primarily for comparing abnormal with normal films; accurate quantitative measurements with it are possible but less satisfactory. The Bock erythrocytometer did not give correct readings on abnormal cells.

Emmons' apparatus is a good one but has never been available commercially. Since the principles originally used by Thomas Young and those he employed are so satisfactory, we have been interested in the development of an inexpensive instrument for the diffractometric measurement of red blood cells. C. A. Hausser and Son have been responsible for the mechanical features of the instrument; I have collaborated and made the calibrations.

The instrument is shown in Fig. 1. The light source is a small standard 50 watt projection lamp, usable on a regular light circuit without a transformer. This is set in the base of the instrument. Above the lamp is an opaque disk with a small central aperture. This light source is surrounded by three sets of smaller apertures, which are large enough to be visible but do not create separate spectra. A slot is provided for holding the slide or cover glass on which the blood film has been made or the chamber if a wet preparation is employed. A rack and pinion is provided for varying the distance of the film from the light source. As the film is moved away from the light source, the colored rings of the spectrum become larger. The position of the film can be thus adjusted until the inner red ring used for the measurement directly overlies the inner circle of small holes (Fig. 2).

The accuracy of measurements of the red blood cells depends greatly on the manner in which the film or suspension of red blood cells is prepared. In making a film the cells may be greatly altered from the natural state. The cells in a good film should be well spread so as to not overlap. The film should not be thin enough to flatten the cells. A dry unstained preparation gives a much more brilliant spectrum than a dry stained preparation. The dried plasma seems to influence the measurements also. We have found that the most satisfactory specimens are those made on cover glasses stained with Wright's stain and mounted in physiologic (0.9 per cent) sodium chloride

solution for measuring. A good film of normal blood, when examined under the microscope, should show the normal central concavity of the red blood cell sharply.

In calibrating the apparatus, one colored ring of the concentric spectra must be selected for overlying the circle of small holes. Pijper thinks the measurement is most accurate with the yellow ring. Emmons selected the inner red ring. We have found this ring most easily read and accurate for all variations in the diameter of the cell. Pijper states that relative variations in the red, yellow, and violet rings indicate anisocytosis and poikilocytosis also; we have not been impressed with the value of such interpretations.

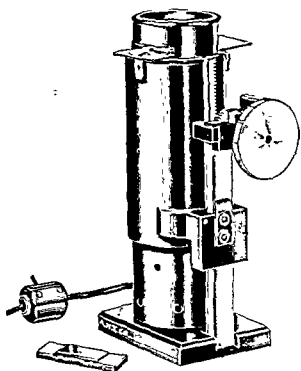


FIG. 1.

FIG. 1.—Haden-Hausser erythrocytometer.

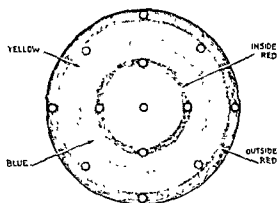


FIG. 2.

FIG. 2.—Field of view of Haden-Hausser erythrocytometer.

Marked anisocytosis and poikilocytosis are apparent on examining the film under the microscope. The great value of the instrument is in determining the mean diameter. The measurement obtained represents the mode or characteristic diameter and not necessarily the mathematical average. Each cell throws its own corona, so the point of maximum intensity corresponds to the figure representing the diameter of the greatest number of cells.

If thought desirable, a fresh wet preparation of red blood cells may be employed. The blood is diluted about 1:200 in a red cell counting pipette with 1.4 per cent sodium oxalate solution which is isotonic with blood. A drop of the diluted blood is placed in a chamber 15 mm. deep, allowed to stand for a few minutes until the cells settle out, covered with a cover glass, and read in the same manner as with the stained specimen. The layer of cells in the chamber is very uniform and the cells are unaltered in the isotonic solution. The spectrum is not so brilliant, however, as with the dry stained preparation mounted in salt solution. The readings are slightly lower in the wet preparation, partly due to the additional thickness of the slide.

We have used the following technique in making the preparations and reading the instrument: Thin, uniform films are made on cover glasses and stained with Wright's stain. The cells in the film should touch but not overlap. A few drops of 0.9 per cent sodium chloride solution are placed on the dry stained preparation, and the cover glass is inverted on a clean glass slide. If the film is made on a slide and stained, an area should be selected for reading where the spread is satisfactory and the shape of the cells is unaltered. A cover glass is mounted over this area with the salt solution. The preparation is moved up and down until the inner red ring is centered over the inner set of holes. The mean cell diameter is read off on the scale located on the wheel used to rotate the pinion.

Three sets of apertures are provided. For reading with the outer set, the second red ring is used; for the middle set, the yellow ring. In a normal blood, the readings should be the same for all three rings and apertures. In an abnormal blood, this is not necessarily true. The measurement of the mean cell diameter is always made with the inner red ring centered over the inner set of apertures.

TABLE I

NUMBER	MEAN CELL DIAMETER BY			DIAGNOSIS
	NEW INSTRUMENT MICRONS	EMMONS' ERIOMETER MICRONS	DIRECT MEASUREMENT MICRONS	
1				
2	7.5	7.6		
3	7.4			
4	7.3	7.4	7.6	Normal
5	7.9	7.4	7.5	Normal
6	7.6	7.9	7.7	Normal
7	7.6	7.6	8.0	Normal
8	7.7	7.6	7.6	Normal
9	6.3	7.6	7.6	Normal
10	6.4	6.3	8.0	Normal
11	6.3	6.4	6.4	Normal
12	6.8	6.4	6.6	Congenital hemolytic icterus
13	6.2	6.8	6.5	Congenital hemolytic icterus
14	7.1	6.3	6.9	Congenital hemolytic icterus
15	7.4	7.1	6.2	Congenital hemolytic icterus
16	8.5	7.3	7.1	Congenital hemolytic icterus
17	8.4	8.6	7.2	Congenital hemolytic icterus
18	8.7	8.4	8.6	Congenital hemolytic icterus
19	8.7	8.7	8.2	Obstructive jaundice
	7.2	8.6	8.9	Obstructive jaundice
20		7.2	8.8	Pernicious anemia
	7.4		6.8	Pernicious anemia
		7.3		Idiopathic hypochromic anemia
			7.2	Idiopathic hypochromic anemia

I have previously shown<sup>a</sup> that the Emmons eriometer gives readings matching closely those obtained with a filar micrometer eyepiece. I have again compared readings obtained with this new instrument and with the Emmons eriometer and by direct measurement using an eyepiece micrometer. The results are shown in Table I. I am under the impression that the results obtained by the diffractometric method are even more reliable than those obtained by direct measurement. Readings are made in a moment. If cover glass preparations are preserved, we have found that the cover glasses are easily removed if heated slightly. After removing the Canada balsam or gum

dammar with xylol and drying, readings are just the same as those originally obtained.

When the diameter is measured directly, a distribution curve (Price-Jones) can be drawn. The shape of the curve shows the degree of anisocytosis. The greatest clinical value of the red blood cell diameter, however, is in the mean diameter, especially when related to the mean red blood cell volume. From these data the thickness can be calculated and the shape of the cell visualized<sup>10</sup>.

#### SUMMARY

An inexpensive, simple, and accurate erythrocytometer for measuring the mean diameter of the red blood cells has been described. A technique for making the preparations for readings which gives a brilliant and sharply read spectrum has been outlined. The routine study of every patient presenting a hematology problem should include the measurement of the mean red blood cell diameter and the calculation of the cell thickness.

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# THE DETERMINATION OF ACETYLSULFAPYRIDINE. I\*

JOHN V. SCUDI AND HARRY J. ROBINSON, RAHWAY, N. J.

WITH the isolation of acetylsulfapyridine from human urine,<sup>1,2</sup> and the discovery that concretions of this substance may cause urolithiasis in experimental animals,<sup>3,4</sup> its determination in body fluids became increasingly important.

The procedure first employed by Marshall<sup>5</sup> for the determination of acetylsulfanilamide has, in many instances, been applied directly, without modification, to the estimation of acetylsulfapyridine. In a recent communication,<sup>6</sup> in which he recommended a coupling component superior to the original alcoholic alpha dimethylnaphthylamine, Marshall cautioned investigators against such usage which may lead to errors of significant proportions. In the present study, certain sources of error have been investigated, and the original Marshall procedure has been adapted to the determination of acetylsulfapyridine in blood and urine.

In their study of the reaction of diazotized sulfanilic acid and dimethylaniline in hydrochloric acid, Goldschmidt and Merz<sup>7</sup> have shown that the reaction mechanism is given by a bimolecular equation if a term for the concentration of hydrochloric acid is included in the usual bimolecular expression; and that neutral salts are without influence on the rate of coupling. The coupling of diazotized sulfapyridine with alpha dimethylnaphthylamine is in close analogy with this work. At various concentrations in 0.1 or 0.2 normal hydrochloric acid, diazotized sulfapyridine couples completely within fifteen minutes. The rate is markedly retarded if the acid concentration is increased.

Fig. 1 shows the coupling rates of the products of hydrolysis of acetylsulfapyridine obtained with 2 normal hydrochloric acid.† After seventy minutes of hydrolysis at water bath temperatures, these solutions were cooled and diluted to the appropriate concentrations of acetylsulfapyridine. The final concentration of hydrochloric acid was 0.2 normal in all cases. Stoichiometrically, complete coupling was obtained only after sixty minutes. Similar results, although slightly less striking, were obtained when the acetylsulfapyridine was hydrolyzed with 0.2 normal hydrochloric acid.

It is evident from these data that significant errors would be introduced if the coupling colors were read within ten to fifteen minutes as in the determination of sulfanilamide.

These findings suggest that acid hydrolysis produces some side reaction which has a retarding influence upon the coupling rate. To investigate this

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†The Evelyn<sup>8</sup> photoelectric colorimeter with filter No. 540 has been used throughout. The results given graphically are typical sets of curves.

phenomenon further, solutions of free sulfapyridine were treated with hydrochloric acid at water bath temperatures for seventy minutes. Various concentrations of hydrochloric acid were used, but after the heat treatment, the solutions were cooled and adjusted to 0.2 mg. per cent free sulfapyridine and 0.2 normal hydrochloric acid. The coupling rates were then measured, and it was observed that the rate of coupling varied inversely with the initial concentration of the acid used. With an initial acid concentration of 2.0 N, more than sixty minutes were required for complete coupling. These data indicate that acid hydrolysis influences the sulfonamide grouping of the free sulfapyridine.

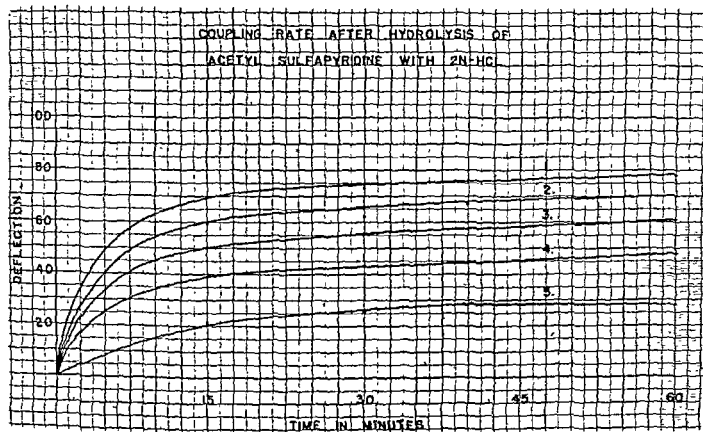


Fig. 1.—Curves 1 through 5 represent concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg. per cent of acetyl sulfapyridine, respectively.

The sulfonamide linkage of sulfapyridine is split by acid hydrolysis. The difference in the coupling rates observed before and after acid hydrolysis can be readily understood on this basis. Meyer<sup>9</sup> has shown that diazotization of 2-aminopyridine in hydrochloric acid yields 2-chloropyridine. Morgan's later work<sup>10</sup> indicates that 2-aminopyridine may be diazotized in the presence of other acids. In the present study a color was observed at high concentrations of 2-aminopyridine, but not at the concentrations actually used. Consequently, it was anticipated that the retardation in coupling rate after acid hydrolysis was due to the liberation of sulfanilic acid which couples more slowly than sulfapyridine under the conditions imposed. A series of pertinent reaction rates is shown in Fig. 2. Acid hydrolysis of sulfapyridine (curve 2) retarded the coupling rate to such an extent that the reaction was complete only after sixty minutes. Mixing stoichiometric amounts of sulfanilic acid and 2-aminopyridine (curve 3) gave the same results, as did an equivalent weight of sulfanilic acid (curve 4). This is strong evidence for the cleavage of the sulfonamide grouping upon acid hydrolysis of sulfapyridine.

To confirm these findings, the absorption spectra of acid solutions of sulfapyridine before and after hydrolysis were compared with that of an equivalent solution containing sulfanilic acid and 2-aminopyridine.\* The results are shown in Fig. 3. Under the conditions used, approximately 80 per cent of the drug was split at the sulfonamide linkage.

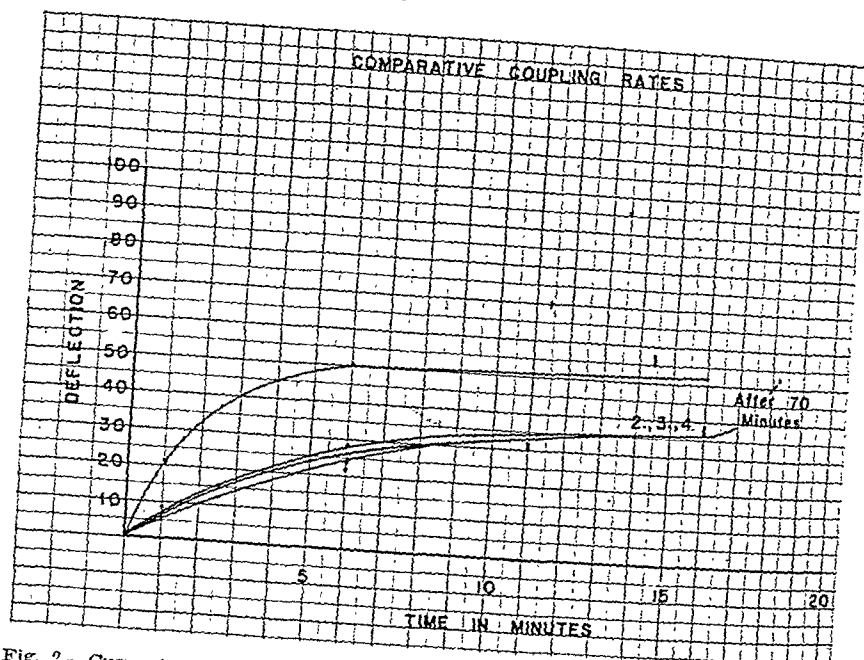


Fig. 2.—Curve 1 shows the coupling rate of 0.2 mg. per cent sulfapyridine diazotized and coupled in 0.2 N HCl. Curve 2 gives the influence of acid hydrolysis on this coupling rate. A solution of 2.0 mg. per cent in sulfapyridine and 2 N in HCl was warmed seventy minutes on a water bath. It was cooled and diluted 1:10 with water. Curve 3 shows the coupling rate of stoichiometric amounts of sulfanilic acid and alpha aminopyridine. Curve 4 gives the coupling rate of an equivalent weight of sulfanilic acid.

The sulfonamide linkage is remarkably stable to alkaline hydrolysis. Thus, using an alkaline hydrolysis of acetylsulfapyridine, complete coupling was obtained within twelve to eighteen minutes. Where it is possible to use an alkaline hydrolysis of acetylsulfapyridine, not only will the sulfonamide linkage remain intact, with a concomitant increased coupling rate, but further, certain interfering reducing substances,<sup>11</sup> such as ascorbic acid, gluco-reductones, thiols, etc., will be removed. Such an alkaline hydrolysis already has been used with the purpose of removing interfering substances in the determination of acetylsulfanilamide.<sup>12</sup> More recently, Baines and Wien<sup>2</sup> have used an alkaline hydrolysis in the determination of acetylsulfapyridine.

*Determination of Acetylsulfapyridine in Urine.*—In a series of 25 normal urine specimens, an average recovery of 103 per cent ( $\pm 2$  per cent, maximum deviation 6 per cent) was obtained when the urine samples containing known amounts of added acetylsulfapyridine were treated as follows: 1 c.c. of urine, 9 c.c. of water, and 5 c.c. 8 normal sodium hydroxide† were warmed on a steam bath for seventy minutes. The reaction mixture was cooled by addition of

\*These data have been furnished by Dr. T. J. Webb and Mr. W. Bastedo of the Research Laboratories of Merck & Co., Inc.

†It is not necessary to reflux the solution as recommended by Baines and Wien,<sup>2</sup> nor is a rigorous standardization of the acid and alkali necessary.

approximately 50 c.c. water. Twelve cubic centimeters of 4 normal hydrochloric acid were added and the volume adjusted to 100 c.c., giving a urine dilution of 1 to 100. At this dilution, urines containing 10 mg. per cent acetylsulfapyridine can be accurately analyzed. At higher concentrations of the drug, greater dilutions may be used. The diazotization and coupling were essentially that described by Marshall.<sup>5</sup> To 10 c.c. of the hydrolyzed and diluted urine, 1 c.c. of

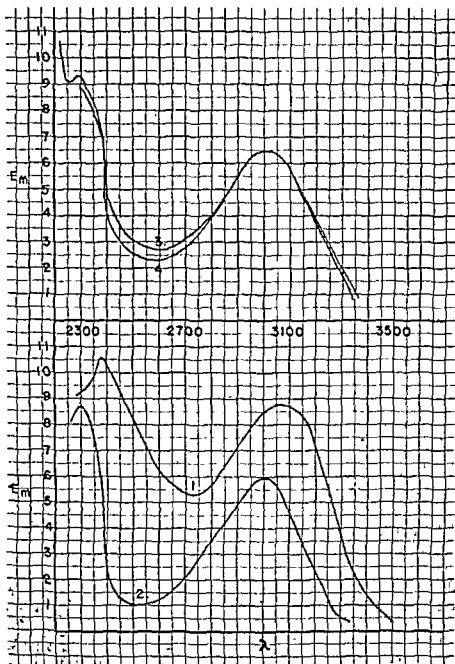


Fig. 3.—Curve 1 gives the absorption data for sulfapyridine in 0.2 N HCl. Curve 2 represents the absorption of an equivalent solution of sulfanilic acid and alpha amino pyridine. Curve 3 shows the absorption of an equivalent solution of sulfapyridine after hydrolysis for seventy minutes in 2 N HCl. (This solution was diluted to 0.2 N and the absorption measured.) Curve 4 was calculated for 80 per cent hydrolysis.

0.1 per cent sodium nitrite was added, and after three minutes, 1 c.c. of 0.5 per cent ammonium sulfamate was added. After two minutes, 5 c.c. of alcoholic dimethylnaphthylamine were added, the contents were mixed by inverting the tube twice, and the colors were read in the colorimeter after fifteen minutes. Very little further change occurred if the tubes were allowed to stand for six hours.

*Determination of Acetylsulfapyridine in Blood.*—The method reported by Marshall for the determination of free sulfanilamide is directly applicable to the estimation of free sulfapyridine in urine and in blood. For blood at a 1:50 dilution, 100 per cent recovery has been reported.



In the present study, water was employed as the laking agent. An acid hydrolysis<sup>5</sup> was employed since some filtrates developed a yellow color during alkaline hydrolysis. Using 0.5 c.c. of 4 normal hydrochloric acid per 10 c.c. of blood filtrate containing varying known amounts of acetylsulfapyridine, the hydrolysis was found to be incomplete and, therefore, 0.5 c.c. of 8 normal hydrochloric acid was used. In order to neutralize the excess acid, 0.7 c.c. of 4 normal sodium hydroxide was added before replacing the water of evaporation. Under these conditions coupling was complete within sixty to seventy minutes. The colors developed were stable for at least six hours.

At a 1:50 dilution, the recovery of added acetylsulfapyridine in a series of 23 determinations was 92 per cent  $\pm$  2 per cent, maximum deviation 6 per cent. Final values should, therefore, be divided by 0.92.

#### SUMMARY

The hydrolysis of acetylsulfapyridine in acidic and alkaline solutions has been studied. Cleavage of both the acetyl and the sulfonamide groupings occurs upon acid hydrolysis. The liberated sulfanilic acid after diazotization couples much more slowly than sulfapyridine. This retardation in the coupling rate causes variable results when the method devised for the determination of acetylsulfanilamide is applied directly to the estimation of acetylsulfapyridine. Since alkaline hydrolysis does not cleave the sulfonamide grouping, this retardation in the coupling rate may be eliminated.

An alkaline hydrolysis gave a recovery of 103 per cent ( $\pm$ 2, maximum deviation 5 per cent) of acetylsulfapyridine added to urine. In blood, 92 per cent ( $\pm$ 2 per cent) of added acetylsulfapyridine was recovered by using an acid hydrolysis.

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## THE DETERMINATION OF ACETYSULFAPYRIDINE. II\*

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RECENTLY Marshall<sup>1</sup> modified his original procedure<sup>2</sup> by replacing the alcoholic alpha dimethylnaphthylamine with a superior beta component, N-(1-naphthyl) ethylenediamine dihydrochloride. The new method was applied to the determination of sulfapyridine, but not to that of acetylsulfapyridine. Since studies in our laboratories<sup>3, 4</sup> required a method for the estimation of this latter substance, Marshall's method has been applied to the estimation of acetylsulfapyridine.

It has been shown<sup>5</sup> that in dilute solution acid hydrolysis of acetylsulfapyridine produces cleavage of both the acetyl and the sulfonamide groupings. The sulfanilic acid so liberated couples with alcoholic alpha dimethyl-naphthylamine much more slowly than sulfapyridine. Consequently, if readings are taken ten to fifteen minutes after coupling, as in the determination of acetylsulfanilamide, appreciable errors are introduced.

The use of N-(1-naphthyl) ethylenediamine dihydrochloride circumvents these rate-retarding factors in a satisfactory, practical way. Under the conditions standardized by Marshall, the coupling of diazotized sulfapyridine to the new beta component is complete within one minute. Complete coupling required five minutes when the hydrochloric acid was increased from 0.2 to 0.8 normal, and three minutes were required when the sulfapyridine was replaced by sulfanilic acid. While these are marked retardations in rate, they are of no technical significance, insofar as the performance of the test is concerned, since the time required for complete coupling is short in either case.

With this newer and more sensitive component, the extension of Marshall's method to the determination of acetylsulfapyridine in blood and urine involves little more than a record of recovery data.

*Blood Determination.*—At a 1:50 blood dilution, with the Evelyn colorimeter with filter No. 540, 100 per cent recovery of added free sulfapyridine (4, 8, 14, and 20 mg. per cent) was obtained, confirming Marshall's data. In the determination of acetylsulfapyridine, 8 normal hydrochloric acid was used to insure complete hydrolysis. Saponin was omitted in this work. The method is essentially that used by Marshall. To 1 c.c. of blood containing known amounts of added sulfapyridine, 41 c.c. of water, and after ten minutes, 9 c.c. of 15 per cent trichloroacetic acid were added. To 10 c.c. of the filtrate, 0.5 c.c. of 8 normal hydrochloric acid was added, and the tube heated in a water bath for seventy minutes. The reaction mixture was cooled, adjusted to 10 c.c., and diazotized and coupled by the newer method, viz., 1 c.c. of 0.1 per cent sodium nitrite was added, and after three minutes, 1 c.c. of 0.5 per cent ammonium sulfamate was

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added. After two minutes, 1 c.c. of 0.1 per cent N-(1-naphthyl) ethylenediamine dihydrochloride was added, and the colors were read fifteen minutes after mixing.

For added concentrations of 4, 6, 10, and 16 mg. per cent acetylsulfapyridine, recovery of 96 per cent ( $\pm 2$ , maximum deviation, 5 per cent) was obtained in a series of 13 experiments at a dilution of 1:50. The final value for acetylsulfapyridine is thus obtained by dividing by 0.96.

*Urine Determination.*—Comparison of acid and alkaline hydrolysis did not show differences in the galvanometer deflections, but on visual comparison a yellow cast was observed in many samples hydrolyzed by acid. An alkaline hydrolysis was, therefore, used, and a recovery of 100 per cent ( $\pm 2$ , maximum deviation, 5 per cent) was obtained when 16 normal urine samples to which acetylsulfapyridine had been added in concentrations of 20, 25, 30, and 40 mg. per cent, were treated as follows: 1 c.c. of urine, 9 c.c. of water, and 5 c.c. of 8 normal sodium hydroxide were heated in a water bath for seventy-five minutes. The reaction mixture was cooled by dilution to approximately 50 c.c., 6 c.c. of 8 normal hydrochloric acid were added, and the volume was adjusted to 100 c.c. Ten cubic centimeters of this hydrolyzed and diluted urine (1:100) were then diazotized and coupled as described above.

#### SUMMARY

Marshall's method for the determination of acetylsulfanilamide, using N-(1-naphthyl) ethylenediamine dihydrochloride as the coupling component, has been applied to the determination of acetylsulfapyridine. Two variations have been introduced; namely, an increase of acid for the hydrolysis of blood filtrates, and an alkaline hydrolysis for urine. Recovery data are recorded.

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# A METHOD FOR RAPID DETERMINATION OF MAGNESIUM IN BODY FLUIDS AND SOME PRELIMINARY RESULTS ON CLINICAL MATERIAL\*

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METHODS are described in the literature for both the chemical and spectrographic determination of magnesium in biologic fluids. If suitable equipment is available, the spectrographic methods have an obvious advantage over the chemical methods. The most suitable spectrographic method would appear to be the dripping arc method described by Thomson and Lee (*J. Biol. Chem.* 118: 711, 1937). An attempt was made to use this method, but so much practical difficulty was encountered that it seemed very desirable to work out something more suitable for routine determinations. The following simple expedient was developed and proved to be eminently satisfactory.

One or 1.5 c.c. of the body fluid were diluted with distilled water to a total volume of 15 c.c. in a flat bottomed glass tube about 1 inch in diameter and  $1\frac{3}{4}$  inches long. This solution was atomized by a fine glass spray jet illustrated in Fig. 1. The fine spray was directed into and on the surface of the electrodes of a carbon spark. The pressure for operating the atomizer was supplied from an oxygen tank through an ordinary reducing regulator which latter kept the pressure on the jet constant. The pressure and the jets were adjusted so that it took two minutes to spray the entire contents (15 c.c.) of the glass tube through the spark. An exposure was made on an Eastman O-III ultraviolet plate in a small quartz spectrograph while the solution was being sprayed through the spark. Under these conditions, with the apparatus used, the magnesium 2795.5 line has just the correct range of density for the usual concentrations of magnesium in whole blood, blood plasma, cerebrospinal fluid, bile, etc. Of course, these fluids are diluted 10 or 15 times before being sprayed, so that, if necessary, a determination could be made by this method on 0.25 c.c. of original fluid instead of the 1 or 1.5 c.c. used in this work.

Calibrating exposures were made on each plate with solutions of magnesium chloride containing 1 mg., 0.5 mg., 0.25 mg. per 100 c.c., respectively. At first an attempt was made to use manganese as an internal standard, but this was not found to be entirely satisfactory, probably due to a large effect of the organic matter on the intensities of the manganese lines. Within the usual range of accuracy of biochemical determinations, the intensity of the magnesium 2795.5 line did not seem to be seriously affected by the organic matter present, although it seemed that much cleaner looking lines could be

\*From the Buhl Memorial Laboratory, Harper Hospital, Detroit.  
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obtained when organic matter was absent. The density of the 2795.5 line was determined with a Hartmann Comparator. The densities for the standard magnesium concentrations were plotted against the logarithms of the concentrations. The three points for the standards were always nearly on a straight line. These straight lines always had nearly the same slope, although their positions varied considerably. Results for the body fluid solutions were used only when the density of the lines fell within the range covered by the standards. The results so obtained were considered to be absolute values, although it is quite possible that although they are relatively correct, they are off absolutely, due to the effect of other substances on the magnesium line. The values obtained ran higher than those reported in the literature as determined by chemical methods. It does not seem possible at present to prove whether

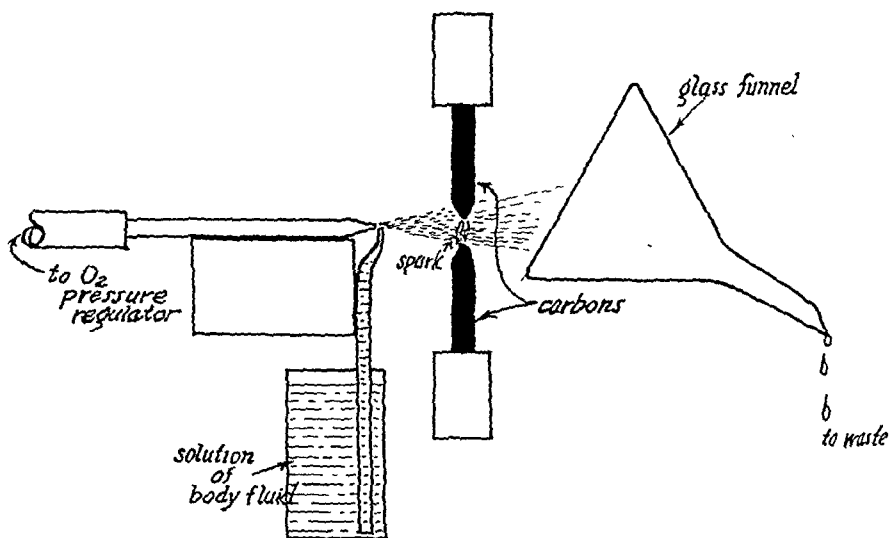


Fig. 1.

this effect is spurious or whether the chemical methods do not show up all the magnesium present as might be the case if organically combined, nonionic magnesium were present in the deproteinized filtrate used in the colorimetric magnesium determinations. It is an interesting speculation to consider that such magnesium compounds are present and play an essential role as catalysts in the inverse reaction to photosynthesis, the formation of carbon dioxide and water from carbohydrates or intermediates. This is in line with the large magnesium content of muscular tissue and with the chemical nature of chlorophyll.

Returning to the method, the carbons are cut wedge shaped, with a rounded-off edge. If the spray is adjusted so that the rounded-off edges are always kept wet by the spray, the same carbons can be used for hundreds of determinations. A check run on distilled water will show only the faintest trace of a magnesium line, even though the run is directly after a high magnesium run. If an exposure is taken with no spray, the magnesium lines show up intensely after the carbons have been used. However, the spray continually

renews the surface to which the spark strikes, so that solutions that have soaked into the carbons previously seem to have a negligible effect even though the carbon lines show up strong.

This method of determining magnesium is very rapid. One operator could possibly make 200 determinations a day if that many samples were available.

The method has been applied in a preliminary way to whole bloods, blood plasmas, and cerebrospinal fluids which have been received in the laboratory of Harper Hospital. Some trichloroacetic acid filtrates were run, but as it was found that satisfactory determinations could be made without removing the protein, the use of the filtrates was abandoned. All the bloods used were oxalated.

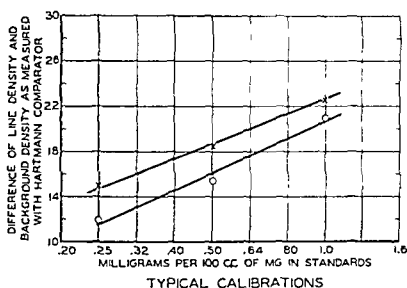


Fig. 2.

As determined by this method, the blood plasma values are 3 to 4 mg. per 100 c.c., while for whole blood we obtain 6 to 7 mg. per 100 c.c. The generally accepted averages are 2.7 mg. per 100 c.c. for blood plasma, and 4 mg. per 100 c.c. for whole blood. As in the chemical determinations, the magnesium does not show a high degree of constancy, but fluctuates considerably. The fluctuation of the spectrographic values does not seem to be as wide as that of the chemical values. On about 50 determinations on human bloods, no strikingly abnormal values of magnesium content have been found. However, the amount of work done so far is very limited.

On cerebrospinal fluids, which generally contain less magnesium than blood plasma, 3 mg. per 100 c.c., as determined by this method, is the average.

One interesting result of 7.5 mg. per 100 c.c. was found in the spinal fluid of an infant ten months old, brought to the clinic with signs of cerebral atrophy and spastic phenomena due to birth anoxia. Aside from the neurologic signs of cortical atrophy, which was confirmed by encephalogram, there was cervical lymph node enlargement, and a high serum phosphatase with normal calcium and phosphorus levels.

It is known that magnesium ions activate phosphatase. Whether the high magnesium was responsible for the high phosphatase or vice versa cannot be told from the available evidence. This case seems to be the first on record of an abnormal magnesium content of the cerebrospinal fluid.

# A MICRODIFFUSION METHOD FOR THE ESTIMATION OF ACETONE\*

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## APPARATUS AND METHOD

QUANTITATIVE determination of varied volatile substances has been made simple by the use of a diffusion apparatus devised by Conway and Byrne<sup>1</sup> in 1933. The apparatus known as the "Conway unit" is on the market.<sup>2</sup> The principle back of it, according to the inventors, "consists in the absorption by simple gaseous diffusion of the volatile substance from one chamber where it exerts a certain tension into another where its tension is zero on the surface of an absorbing fluid." Workers have previously made use of the principle, but their technique did not permit reasonably rapid results (Durig,<sup>3</sup> Neuberg<sup>4</sup>).

The unit consists of a small dish of fairly thick glass, resembling a Petri dish. It is divided into two chambers by a circular wall of glass arising from the floor of the dish to about half the height of the outer wall. When in use it is covered by a ground-glass plate and sealed with a fixative.

Since the introduction of the unit, which was associated with a plan for the microdetermination of ammonia, other reactants have been studied with it: chloride and bromide (Conway and Flood<sup>5</sup>); urea (Conway<sup>6</sup>); acetone and alcohol (Fearon and Webb<sup>7</sup>). A review of the literature is not intended here, but it may be mentioned that Professor Conway's book entitled *Quantitative Analysis by the Micro-Diffusion Method*<sup>8</sup> was recently published. This book deals with the procedures developed with the unit, and their various applications so far considered.

The method for the estimation of acetone makes use of the Conway unit and the recognition of acetone by means of Nessler's solution (Deniges<sup>9</sup>).

## TECHNIQUE

In this method for the estimation of acetone one considers the time necessary to cause precipitation of the Nessler's solution, and the temperature at which the reaction takes place. For accurate results the exact temperature and time concerned are important. In this work temperature was fairly well controlled by incubation of the apparatus and reagents before using, and a stop watch was used to obtain the time.

The reagents and their amounts are as follows: 2 c.c. of Nessler's solution, 3 c.c. of unknown, and 1 c.c. of dilute acid. The first is introduced into the inner chamber of the unit, and the latter two into the outer. The acid is used

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to fix any ammonia which may be present in the unknown. The observed end point is the appearance of precipitation of the Nessler's solution at the periphery of the inner chamber in the form of threads, like spokes of a wheel, or as a cloud. It is that second when the spokes or cloud extend inward 1 mm. This end point was chosen in order to establish uniformity. To permit visibility, a transparent glass plate is used here instead of a ground glass one suggested for other methods.

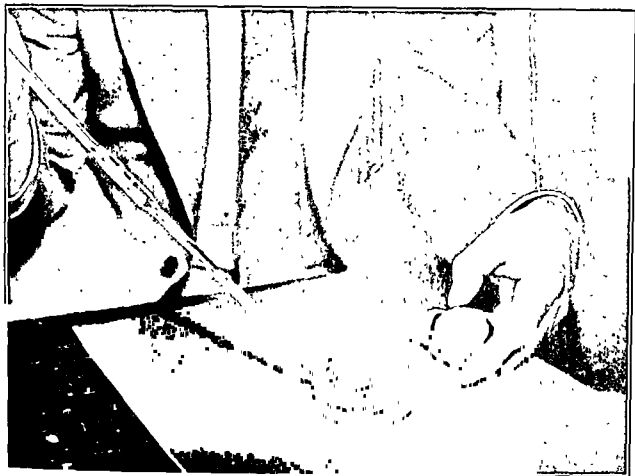


Fig. 1.

The procedure followed involved primarily the careful cleaning of the units. Careful handling is necessary to avoid chipping of the edges, and proper washing eliminates traces of acetone. Chipping spoils the apparatus, for it needs to be hermetically sealed, and a trace of acetone would interfere with the end point, because of the sensitivity of the Nessler's solution. After washing the units in the usual way with soap and water, it was found best to soak them overnight in a large beaker containing weak cleaning solution. Before using they were thoroughly rinsed in tap and distilled water, and dried after dipping in alcohol and ether. Drying is facilitated when the units are placed in the incubator.

When ready for use, the units with their covers were placed on a table spread with black paper (a dark background helps in the reading of the end point), and the edges of the outer wall smeared with vaseline. Next the reagents are introduced. First the central chamber received the Nessler's solution, and then the acid was run in, distributing it evenly around the outer chamber. The transparent glass plate was next placed to cover the entire unit, except for a small slit, permitting the introduction of the end of the pipette used to run in the unknown. A stop watch held in one hand, and a pipette containing the unknown in the other, is started as soon as the pipette



stops flowing freely. The hand holding the watch next slides the glass plate so that it completely covers the unit as an immediate gesture after starting the watch (Fig. 1). When the end point is reached, the stop watch is stopped.

### RESULTS

*The Effect of Concentration.*—To study the effect of concentration, dilutions of "specified" acetone in distilled water were prepared and incubated at 16° C. Time readings for these tests, as well as for the others in this paper, were repeated and checked by three individuals, in order to eliminate personal error, and to observe the influence of practice. Table I shows the average readings obtained using dilutions ranging from 1:100 to 1:10,000. Upon plotting concentration against time it was observed that a parabolic-like curve was formed. Applying the parabolic formula, and using an average constant, obtained by averaging the constants given by several diverse dilutions, comparable figures resulted. Substituting into the parabolic formula our letters, the formula  $t^2/D = K$  resulted, with  $t$  representing time,  $D$  dilution, and  $K$  the constant.

TABLE I

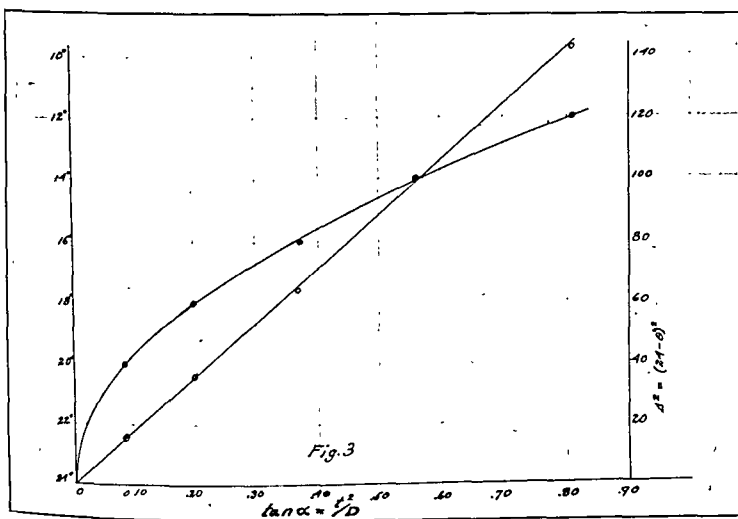
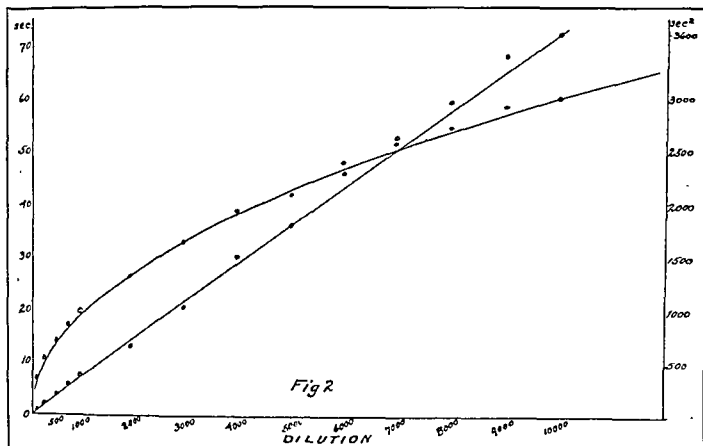
DILUTIONS (1:100 = 1% SOLUTION)	TIME (IN SECONDS)
1: 100	7.0
1: 250	11.0
1: 500	14.5
1: 750	17.5
1: 1,000	20.0
1: 2,000	26.5
1: 3,000	33.0
1: 4,000	39.0
1: 5,000	42.0
1: 6,000	48.0
1: 7,000	51.5
1: 8,000	54.5
1: 9,000	58.5
1:10,000	60.0

TABLE II  
TEMPERATURE

DILUTIONS	12° c.	14° c.	16° c.	18° c.	20° c.
1: 100	9	8	7	5	3
1: 1,000	30	25	20	13	11
1:10,000	90	75	60	47	32

*The Effect of Temperature.*—To study the effect of temperature, dilutions of 1:100, 1:1,000, and 1:10,000 were used and incubated at a number of temperatures. Table II shows the relationship of the time factor in average figures obtained with these dilutions at five temperatures, two Centigrade degrees apart. A study of this table indicates the regularity in the relationship between temperature and time. This regularity has been incorporated in the following formula, which includes concentration, temperature, and time:

$$D = \frac{179 t^2}{(24 - \Theta)^2} \text{ where } D = \text{dilution, } t = \text{time, and } \Theta = \text{temperature.}$$



*The Effect of Colloids.*—Ox blood and milk were used to study the effect of colloids. Dilutions of these fluids were made as in the above experiments, and tested in the same way. Proportions of acetone in them gave time readings, with changes in concentration and temperature, similar to those obtained by the same proportions of acetone in water.

## DISCUSSION

Each of the time values given in the tables in seconds is the average of six to ten readings by three observers, for which the total variation is not more than three seconds. The relative deviations naturally are smaller for the slower times.

In Fig. 2 the average reading obtained for known dilutions ranging between 1:100 and 1:10,000 at 16° C. are indicated by small circles near the parabola drawn for the equation  $t^2 = 0.35 D$ . The straight line in this figure shows the same relationship when the square of time is plotted against dilution. The actual readings are similarly indicated near to the straight line.

## NOMOGRAPHIC CHART

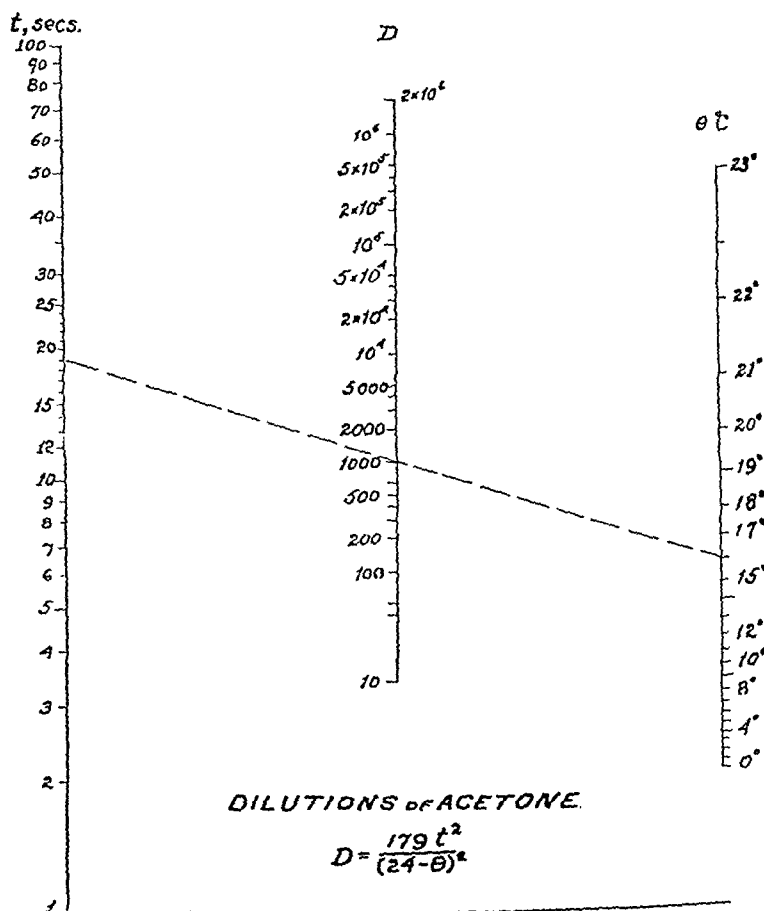


Fig. 4.

In order to determine whether a constant factor for temperature exists, similar graphs were made for results at temperatures from 12° to 20° C. The slopes of these lines, expressed as tangents, are given with their corresponding temperatures in Table III. These values also fall along a parabola, as shown in Fig. 3. The parabola in this figure is drawn for the equation  $A^2 = 179 \tan \gamma$ , and the straight line expresses the same relationship, values

of  $\Delta^2$  being used as ordinates. The points indicated by small circles are plotted for the values in Table III, of  $\tan \gamma = t^2/D$ , and  $\Delta$ , equal to the difference between  $24^\circ$  C. and the observed temperature of the reaction. The combined equation, expressing the relationship between dilution ratio, time in seconds, and temperature in degrees Centigrade, then, is  $D = \frac{179 t^2}{(24^\circ - \Theta)^2}$

It is evident from this equation that as the temperature approaches  $24^\circ$  C., the reaction rate becomes immeasurably fast. The temperature  $20^\circ$  C. is the highest at which, up to the present time, it has been found possible to obtain concordant results.

TABLE III

$\Theta$ (DEGREES C.)	$\Delta =$ $24^\circ - \Theta$	$\Delta^2$	$\tan \gamma =$ $t^2/D$	$K =$ $\Delta^2/\tan \gamma$
$12^\circ$	$12^\circ$	144	0.81	178
$14^\circ$	$10^\circ$	100	0.56	179
$16^\circ$	$8^\circ$	64	0.36*	178
$18^\circ$	$6^\circ$	36	0.20	180
$20^\circ$	$4^\circ$	16	0.09	178

\*The value 0.36 is the average ratio for times longer than twenty seconds.

In order to facilitate rapid estimation of dilution ratios, a nomographic chart has been constructed, and is shown in Fig. 4. A black thread or, better, a straight line ruled on a strip of transparent material may be used in aligning the corresponding quantities. The broken line in the figure connects the time, 19 seconds, and the temperature,  $16^\circ$ , and cuts the D scale (dilution ratio) at 1,000.

The empirical constant, 179, does not represent a high degree of precision, but it may vary by several units without seriously affecting the results. The analytical procedure, which has been described, is suitable for the rapid estimation of small concentrations of acetone, and is sufficiently exact within the range of concentrations in which this substance is encountered in physiologic materials.

#### CLINICAL APPLICATION

Fairly accurate results may be obtained by permitting the units and reagents to stand at room temperature, and using it in the proper formula or in the nomographic chart. Until this method will have received adequate testing by others, temperatures above  $20^\circ$  C. may not be considered practical, for the time factor becomes small, introducing greater possible error. At the lower temperatures the time readings from three tests may be averaged and considered sufficient.

The skill necessary to establish uniformity, and to permit reasonably rapid results, is developed with very little practice. Once the end point is observed it is easily remembered, and if the precautions indicated under technique are carried out, no difficulty should be encountered.

A considerable number of specimens of urine and blood, normal and pathologic, have been examined by this method. These tests indicate that normal blood and urine contain free acetone in minute amounts, for precipitation of the Nessler's solution occurred only after hours and in a very thin

film. Whenever acetone was present in pathologic quantities, reasonable time readings were obtained. It may be interesting to note that in a number of cases of diabetes mellitus, with clinical evidence of deep acidosis, the time readings obtained at 16° C., with blood samples, were around one minute. This represents a dilution of about 1:10,000. One of these cases died the day after the examination.

#### CONCLUSIONS

1. A microdiffusion method for the estimation of acetone which may be applied to biological fluids is described.

2. This method lends itself to clinical application due to its simplicity and rapidity of action.

I am grateful to Professor Horatio Hughes, of the College of Charleston, for his assistance in the derivation of the general formula and the formation of the nomographic chart.

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### THE USE OF HONEY AS A LEVULOSE TOLERANCE TEST\*

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THE advent in recent times of various specific methods for the determination of blood levulose make possible the study of levulose tolerance test with greater accuracy than by determining the total blood sugar as it was done before.

Such specific methods for the determination of blood levulose are based on the color reactions of this sugar with diphenylamine, Ihl and Perchmann's reaction; with resorcinol, Selivanoff's reaction; or with sodium tauroglycocholate, according to Scott.

In 1927 Kronenberger and Radt<sup>1</sup> proposed a method for blood levulose determination, starting from Selivanoff's reaction. In the same year, van Creveld<sup>2</sup> presented a new method, this time based on the reaction with

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diphenylamine. This method was the object of various modifications which aimed to make it more accurate.<sup>3-7</sup>

The utilization of resorcinol in the methods of blood levulose determination has been less explored. Besides Kronenberger and Radt, only Roe<sup>8</sup> thought of this possibility. Roe's method,<sup>8</sup> which we had occasion to employ in the present work, offers many advantages over the others, by its simplicity, rapidity, and great specificity. With reference to this last cited advantage, we carried on various tests which confirmed this quality.

Scott's method,<sup>9</sup> based on the color reaction with sodium tauroglycolate, though accurate, has some serious difficulties: it requires 5 c.c. of blood and takes considerable time and work.

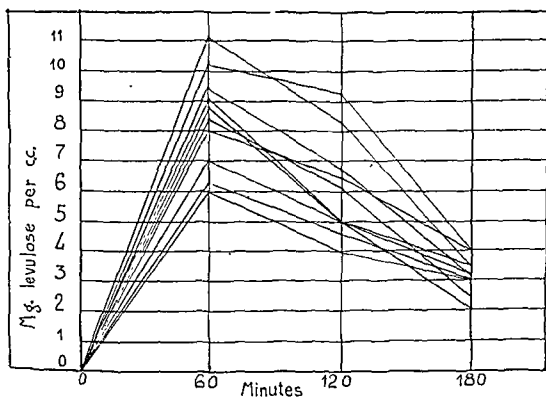


FIG. 1.

During the execution of this present work a new modification of the diphenylamine method was published by Herbert,<sup>10</sup> which eliminated many causes of error in the similar methods, and made this one more practical and specific.

Baeta Vianna,<sup>11</sup> Professor of Biochemistry at the University of Minas Gerais, Brazil, suggested to us the carrying on of levulose tolerance tests but using, instead of levulose itself, honey which contains 40 per cent of this glucide.<sup>12</sup> From determinations which we made with honey, obtained from various sources, we showed that there is always present at least 40 per cent of levulose.

*Method.*—We made the tests on 10 normal persons, all students, in the following way: In the morning, after at least eight hours of fasting, 100 gm. of honey were taken (equal to 40 gm. of levulose), followed by a glass of water. Blood was taken before the intake of honey, and at intervals of one, two, and three hours after taking the honey. Levulose was determined according to Roe's method.<sup>8</sup> Incidentally we determined also the total blood sugar in 5

of our patients. The determination was made by the Hagedorn-Jensen method. In these 5 cases we secured blood before the intake of honey and at the end of the first half hour, and the first, second, and third hours. The glyceimic curve was calculated by the difference. The results are summarized in Table I.

TABLE I

SUBJECTS	MG. PER 100 C.C. BLOOD														
	TOTAL SUGAR					LEVULOSE					GLUCOSE				
	HOURS AFTER INGESTION					HOURS AFTER INGESTION					HOURS AFTER INGESTION				
	0	½	1	2	3	0	½	1	2	3	0	½	1	2	3
1	87	119	84	80	80	0	7	8	6.5	2.5	87	112	76	73.5	77.5
2	87	110	96	69	69	0	7.5	8.5	5	3.5	87	102.5	87.5	64	65.5
3	85	110	96	79	76	0	8	9	5	3	85	102	87	74	73
4	92	122	92	88	79	0	5	6	4.5	3	92	117	86	84	76
5	90	140	130	88	84	0	7	8.5	6	3	90	133	121.5	82	81

## RESULTS

In fast the levulose of the blood is zero; an hour later figures showing between 6 and 11 mg. per cent were obtained; in the second hour, 4.0 and 9.5 mg. per cent; in the third hour, 2 and 4 mg. per cent. Table II and Fig. 1 show all the figures obtained.

TABLE II

SUBJECTS	MG. LEVULOSE PER 100 C.C. BLOOD			
	HOURS AFTER INGESTION			
	0	1	2	3
1	0	8	6.5	2.5
2	0	8.5	5	3.5
3	0	9	5	3
4	0	6	4.5	3
5	0	8.5	6	3
6	0	7	5	2
7	0	11	8	3
8	0	9	7	3
9	0	10	9.5	4
10	0	6	4	3

Our values are like those of Scott.<sup>13</sup> They are somewhat different from those of Stewart and his co-workers<sup>14</sup> owing to the difference in methods employed. They used the method of Patterson,<sup>7</sup> which, according to Herbert,<sup>10</sup> furnishes high values in levulose, due to the interference of glucose. Scott,<sup>13</sup> as well as Stewart and his associates,<sup>14</sup> used pure levulose in their investigation.

## CONCLUSION

The employment of honey (for the levulose that it contains) as a levulose tolerance test, from the curves for levulose obtained and given the figures, identical with those of Scott,<sup>13</sup> when he used pure levulose, suggests its employment as a liver function test, instead of the use of pure levulose, which deserves our attention at the present and whose results will be published soon.

The advantages of the employment of honey as a levulose tolerance test are numerous. The greatest is its low price: the quantity of honey for a test is about 30 times cheaper than pure levulose (Brazil prices). Besides this,

in the tests made, in no case was there evidence of vomiting, nausea, or diarrhea, which is customary in the use of levulose.<sup>15</sup> Therefore, it is customary to demand the very purest levulose to avoid intestinal phenomena which may be produced by the presence of impurities. Honey gets around this difficulty, because the dose administered is quite well tolerated by the individual.

#### SUMMARY

1. The literature was reviewed with reference to methods of determining the levulose in the blood.

2. Curves for levulosemia were obtained from 10 normal individuals after the intake of honey.

3. These findings suggest the use of honey as a levulose tolerance test for the examination of hepatic function.

I wish to thank Professor J. Baeta Vianna for his advice, and for the possibility he granted me of accomplishing this piece of work. My thanks are extended to Professor C. A. Baker and my wife who helped me in the translation of the text from Portuguese into English.

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# UNIFORMITY IN THE DIFFERENTIAL ENUMERATION OF LEUCOCYTES\*

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VARIOUS techniques of making the differential count have been advocated. Although most authorities agree that the counting of every leucocyte on a cover slip preparation is the ideal method, this is much too cumbersome for clinical work. Therefore, smears are commonly made on glass slides and, after staining, the percentage of each leucocyte is determined. Gradwohl<sup>1</sup> recommends counting 100 to 200 leucocytes by the four-field meander method since large cells tend to accumulate near the margins of the smear. Todd and Sanford<sup>2</sup> specify the counting of 500 to 1,000 cells for accuracy but state that 300 cells are sufficient for approximate results. They also regard it as imperative that cells in all parts of the smear be counted, but they give no method of sampling. Kolmer and Boerner<sup>3</sup> recommend counting 100 cells if the total white count is 10,000 or less; 200 cells if the total count is between 10,000 and 15,000, and correspondingly larger numbers as the total count increases. Cummer<sup>4</sup> specifies the counting of 250 cells at least and adds that representative areas from the preparation should be chosen. Kilduffe<sup>5</sup> states that the counting of 200 cells is sufficient for ordinary purposes and that a differentiation of cells within the counting chamber is not a practicable procedure due to difficulties in the preparation of a staining and diluting fluid in one.

In an attempt to determine the relative values of these techniques of differential counting, a series of experiments was performed in which each technique was used in counting the same blood sample and the results compared.

## EXPERIMENTAL

*Counting Chamber Diluting Fluid.*—Preliminary trials using various concentrations of crystal violet, safranine, methylene blue, and brilliant green indicated that crystal violet was taken up preferentially by the leucocyte nucleus to a greater degree than the other dyes used. Preliminary experiments indicated also that concentrations of acetic acid of from 1 per cent to 50 per cent were satisfactory as diluting fluids with these dyes. For the purpose of this work a 0.2 per cent solution of methyl violet 3 B in 1 per cent acetic acid was used, and pipettes were shaken for three minutes in the Kahn shaker at 180 oscillations per minute. This stain enables a differentiation of polymorphonuclears from lymphocytes with fair accuracy but makes no differentiation between types of polymorphonuclear leucocytes.

\*From the Department of Student Health, Southwestern State College, Weatherford. Received for publication, May 15, 1939.

*Blood Specimens and Smears.*—Blood was obtained by venous puncture and oxalated with 2 mg. potassium oxalate per cubic centimeter of blood. Smears were prepared at once on slides using a spreader slide made as follows: A block of wood about  $1\frac{1}{2}$  inches thick was cut in the form of a right triangle 4 inches on a side. A wide notch, parallel with the long dimension and about  $\frac{1}{4}$  inch deep, was then cut in one side (the base), and a similar notch in the hypotenuse. Thus, when the block was placed on a flat surface and a slide placed in each notch, the two slides form a  $45^\circ$  angle with each other. Upon sliding the block along a flat surface, the slide on the hypotenuse traverses the surface of the lower slide, maintaining a constant angle and constant pressure. In practice, it was found desirable to hold the slide on the hypotenuse to the block with a rubber band.

By means of this spreader and definite amounts of blood, it is possible to prepare nearly uniform smears. In the data reported in this paper, 0.01 c.c. of blood was used for each preparation.

*Methods of Enumeration.*—Smears were prepared and stained with Wright's stain in the usual way. A counting chamber (Spencer Bright Line) was filled at the same time, using the diluting fluid described above. A differential count from the counting chamber, made with the high, dry lens, was compared with those obtained by the four-field meander method and by ordinary random selections.

In the tables presented the "random method" refers to the counting of cells from one side of the preparation to the other and back until the required numbers were counted. No cell was counted twice in this technique. In the four-field meander method, the first 25 cells seen at each corner of the slide were recorded and totaled for the 100 cell count; a different area was then chosen for the 200 cell count. Figures for 300 cells by this method represent the arithmetic mean or average of the 300 different cells.

TABLE I  
DATA OBTAINED FROM BLOOD SAMPLE A

NO.	NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHO-CYTE %	LARGE LYMPHO-CYTE %	MONO-CYTE %	EOSINO-PHILE %	BASO-PHILE %
1	100	Random	61.0	29.0	7.0	3.0	0.0	0.0
2	100	Random	59.0	35.0	3.0	2.0	0.0	1.0
3	100	Random	60.0	33.0	3.0	3.0	1.0	0.0
4	100	Random	61.0	33.0	3.0	2.0	1.0	0.0
5	100	Random	58.0	41.0	1.0	0.0	0.0	0.0
6	100	Random	52.0	39.0	7.0	2.0	0.0	0.0
7	100	Random	61.0	28.0	8.0	2.0	1.0	0.0
8	100	Four-field	64.0	31.0	1.0	3.0	0.0	1.0
9	200	Four-field	60.0	31.5	4.5	2.5	1.0	0.5
10	134	Chamber	69.7	26.6	3.8	0.0	0.0	0.0
11	139	Chamber	67.8	26.6	3.4	2.1	0.0	0.0

Table I shows the differential enumeration for 7 random leucocyte counts of 100 cells each, two four-field meander counts comprising a total of 300 cells and two chamber counts of 134 and 139 cells, respectively, on blood sample A. Random count No. 6 was made near the outer margin of the smear.

For convenience in comparison, averages and medians are grouped in Table II. The median represents the figure on each side of which half the values fall. In computing the counting chamber average, equal weight was given each chamber.

TABLE II  
COMPARISON OF DATA FROM BLOOD SAMPLE A

NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHO-CYTE %	LARGE LYMPHO-CYTE %	MONO-CYTE %	EOSINO-PHILE %	BASO-PHILE %
700	Random average	58.8	34.0	4.6			
700	Random median	60.0	33.0	3.0	2.0	0.5	0.1
100	Four-field average	64.0	31.0	1.0	3.0	0.0	0.0
200	Four-field average	60.0	31.5	4.5	2.5	1.0	1.0
300	Four-field average	61.3	31.3	3.3	2.7	0.0	0.5
273	Chamber	68.8	26.6	3.6	1.0	0.0	0.6
							0.0

There are 21 possible combinations of the 7 random counts of 100 cells, taken two at a time. The average values for these combinations, together with the average values for the 35 possible combinations of these 7 counts taken 3 at a time, are shown in Table III. All values are stated in terms of significant figures.

Table IV shows the range between the lowest and highest value for 100 cells and all possible combinations of 200 and 300 cells by the random method for neutrophiles, small and large lymphocytes. The standard deviation (S.D.) is computed from the formula:

$$S.D. = \sqrt{\frac{\sum d^2}{n}}$$

where  $\sum d^2$  is the sum of the squares of the deviations from the arithmetic mean and  $n$  is the number of values. The probable error (P.E.) is computed from the formula:

$$P.E. = 0.67449 S.D.$$

It is apparent from Table IV that the range, standard deviation, and probable error decrease as the number of cells counted increases but that it is necessary to count 300 cells in order to approximately halve the values obtained by counting 100 cells.

A comparison of data from Table II shows that the neutrophile percentage of 100 cells counted by the four-field method is within the range of random 100 cell counts but that the deviation from the mean is greater than one S.D., i.e., 68.26 per cent of all random 100 cell counts should show as good or better accuracy. In fact, errors as great as 5 per cent should occur only about 10 per cent of the time, indicating that this count is not as good as 90 per cent of random counts.

In counts of 200 cells by the four-field method, a deviation of 1.2 for neutrophiles, as compared with the standard deviation of 1.9 and the probable error of 1.3 for random 200 cell counts, indicates that about 50 per cent of all random counts should show the same accuracy.

TABLE III

AVERAGE VALUES FOR ALL COMBINATIONS OF 200 AND 300 LEUCOCYTES BY THE RANDOM METHOD

RANDOM COUNT NUMBER	NEUTROPHILE %	SMALL LYMPHO- CYTE %	LARGE LYMPHO- CYTE %	MONOCYTE %	EOSINOPHILE %	BASOPHILE %
1, 2	60.0	32.0	5.0	2.5	0.0	0.5
1, 3	60.5	31.0	5.0	3.0	0.5	0.0
1, 4	61.0	31.0	5.0	2.5	0.5	0.0
1, 5	59.5	35.0	4.0	1.5	0.0	0.0
1, 6	56.5	34.0	7.0	2.5	0.0	0.0
1, 7	61.0	28.5	7.5	2.5	0.5	0.0
2, 3	59.5	34.0	3.0	2.5	0.5	0.5
2, 4	60.0	34.0	3.0	2.0	0.5	0.5
2, 5	58.5	38.0	2.0	1.0	0.0	0.5
2, 6	55.5	37.0	5.0	2.0	0.0	0.5
2, 7	60.0	31.5	5.5	2.0	0.5	0.5
3, 4	60.5	33.0	3.0	2.5	1.0	0.0
3, 5	59.0	37.0	2.0	1.5	0.5	0.0
3, 6	56.0	36.0	5.0	2.5	0.5	0.0
3, 7	60.5	30.5	5.5	2.5	1.0	0.0
4, 5	59.5	37.0	2.0	1.0	0.5	0.0
4, 6	56.5	36.0	5.0	2.0	0.5	0.0
4, 7	61.0	30.5	5.5	2.0	1.0	0.0
5, 6	55.0	40.0	4.0	1.0	0.0	0.0
5, 7	59.5	34.5	4.5	1.0	0.5	0.0
6, 7	56.5	33.5	7.5	2.0	0.5	0.0
1, 2, 3	60.0	32.3	4.3	2.7	0.3	0.3
1, 2, 4	60.3	32.3	4.3	2.3	0.3	0.3
1, 2, 5	59.0	35.0	3.7	1.7	0.0	0.3
1, 2, 6	57.3	34.3	5.7	2.3	0.0	0.3
1, 2, 7	60.3	30.7	6.0	2.3	0.3	0.3
1, 3, 4	60.7	31.7	4.3	2.7	0.7	0.0
1, 3, 5	59.7	34.3	3.7	2.0	0.3	0.0
1, 3, 6	57.7	35.7	5.7	2.7	0.3	0.0
1, 3, 7	60.7	30.0	6.0	2.7	0.7	0.0
1, 4, 5	60.0	34.3	3.7	1.7	0.3	0.0
1, 4, 6	58.0	33.7	5.7	2.3	0.3	0.0
1, 4, 7	61.0	30.0	6.0	2.3	0.7	0.0
1, 5, 6	57.0	36.3	5.0	1.7	0.0	0.0
1, 5, 7	60.0	32.7	5.3	1.7	0.3	0.0
1, 6, 7	58.0	32.0	7.3	2.3	0.3	0.0
2, 3, 4	60.0	33.7	3.0	2.3	0.7	0.3
2, 3, 5	59.0	36.3	2.3	1.7	0.3	0.3
2, 3, 6	57.0	35.7	4.3	2.3	0.3	0.3
2, 3, 7	60.0	32.0	4.7	2.3	0.7	0.3
2, 4, 5	59.3	36.3	2.3	1.3	0.3	0.3
2, 4, 6	57.3	35.7	4.3	2.0	0.3	0.3
2, 4, 7	60.3	32.0	4.7	2.0	0.7	0.3
2, 5, 6	56.3	38.3	3.7	1.3	0.0	0.3
2, 5, 7	59.3	34.7	4.0	1.3	0.3	0.3
3, 4, 5	59.7	35.7	2.3	1.7	0.7	0.0
3, 4, 6	57.7	35.0	4.3	2.3	0.7	0.0
3, 4, 7	60.7	31.3	4.7	2.3	1.0	0.0
3, 5, 6	56.7	37.7	3.7	1.7	0.3	0.0
3, 5, 7	59.7	34.0	4.0	1.7	0.7	0.0
3, 6, 7	57.7	33.3	6.0	2.3	0.7	0.0
4, 5, 6	57.0	37.7	3.7	1.3	0.3	0.0
4, 5, 7	60.0	34.0	4.0	1.3	0.7	0.0
4, 6, 7	58.0	33.3	6.0	2.0	0.7	0.0
5, 6, 7	57.0	36.0	5.3	1.3	0.3	0.0

TABLE IV

STATISTICAL SUMMARY OF RANDOM COUNTS FROM BLOOD SAMPLE A

NO. OF CELLS IN COMBINATION	NEUTROPHILES			SMALL LYMPHOCYTES			LARGE LYMPHOCYTES		
	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.
100	9.0	3.0	2.0	13.0	4.4	3.0	7.0	2.5	1.7
200	6.0	1.9	1.3	12.5	2.9	1.9	5.5	1.6	1.1
300	4.4	1.4	1.0	8.3	2.1	1.4	5.0	1.2	0.8

No significant comparison can be made between random 300 cell counts and the four-field average, since the error shown in the 100 cell four-field count is carried over into the average for that technique.

The 10 per cent neutrophile deviation in the counting chamber method is not only outside the range of all random cell counts but since the ratio of the deviation to the standard deviation of the 200 cell count is approximately 5, the odds against this great a deviation in random counting are at least  $1.7 \times 10^6$  to 1<sup>6</sup>. It may be noted, however, from Table II that the sum of neutrophiles and small lymphocytes approximates a similar summation from random counts, thus indicating mistaken identification rather than improper distribution.

The close agreement of the median and mean in Table II would indicate that 700 cells represents a thorough sampling of the slide and that the arithmetic mean is, therefore, close to the true value.

Random count No. 6, made at the margin of the smear, does not indicate that large cells tend to accumulate near the margins.

Table V shows the differential enumeration of 10 random counts of 100 cells each, two four-field meander counts comprising a total of 300 cells and one chamber count of 189 cells on blood sample B. Random count No. 10 was made at the margin of the smear. For convenience in comparison, averages and medians are grouped in Table VI, and a statistical summary is shown in Table VII. In the preparation of Table VII, 45 possible combinations of the 10 random counts taken two at a time were averaged as were 120 possible combinations of the 10 random counts taken three at a time. Calculations were made as explained for Tables III and IV.

TABLE V  
DATA OBTAINED FROM BLOOD SAMPLE B

NO.	NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHOCYTE %	LARGE LYMPHOCYTE %	MONOCYTE %	EOSINOPHILE %	BASOPHILE %
1	100	Random	56.0	35.0	5.0	2.0	1.0	1.0
2	100	Random	52.0	47.0	1.0	0.0	0.0	0.0
3	100	Random	59.0	38.0	2.0	1.0	0.0	0.0
4	100	Random	59.0	38.0	3.0	0.0	0.0	0.0
5	100	Random	54.0	40.0	5.0	1.0	0.0	0.0
6	100	Random	58.0	41.0	1.0	0.0	0.0	0.0
7	100	Random	48.0	49.0	2.0	1.0	0.0	0.0
8	100	Random	48.0	48.0	2.0	2.0	0.0	0.0
9	100	Random	50.0	48.0	2.0	0.0	0.0	0.0
10	100	Random	35.0	61.0	1.0	3.0	0.0	0.0
11	100	Four-field	63.0	34.0	0.0	3.0	0.0	0.0
12	200	Four-field	49.0	48.5	1.0	1.0	0.5	0.0
13	189	Chamber	56.5	36.6	4.8	2.2	0.0	0.0

TABLE VI  
COMPARISON OF DATA FROM BLOOD SAMPLE B

NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHOCYTE %	LARGE LYMPHOCYTE %	MONOCYTE %	EOSINOPHILE %	BASOPHILE %
1000	Random average	51.9	44.5	2.4	1.0	0.1	0.1
1000	Random median	53.0	44.0	2.0	1.0	0.0	0.0
100	Four-field	63.0	34.0	0.0	3.0	0.0	0.0
200	Four-field	49.0	48.5	1.0	1.0	0.5	0.0
300	Four-field	53.7	43.7	0.7	1.7	0.7	0.0
189	Chamber	56.5	36.6	4.8	2.2	0.0	0.0

TABLE VII  
STATISTICAL SUMMARY OF RANDOM COUNTS FROM BLOOD SAMPLE B

NO. OF CELLS IN COMBINATION	NEUTROPHILES			SMALL LYMPHOCYTES			LARGE LYMPHOCYTES		
	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.
100	24.0	6.9	4.6	26.0	7.3	4.9	4.0	1.4	1.0
200	17.5	4.6	3.1	19.0	5.0	3.3	4.0	1.0	0.6
300	15.0	3.5	2.3	15.7	3.7	2.5	3.3	0.7	0.5

It is apparent from Table VII that the range, standard deviation, and probable error are inversely proportional to the number of cells counted, although the values show much greater deviation than those from blood sample A. In order to halve the probable error of this 100 cell count it is necessary to count 300 cells.

Table VI shows that in the case of neutrophiles, the four-field method differs from the mean by 11.1 per cent for 100 cells and by 2.9 per cent for the 200 cell count. The ratio of the deviation to the probable error indicates that a deviation this great should occur only 11 per cent of the time by random counting, while the deviation of the 200 cell count by the four-field method is well within the limits of the probable error, i.e., this count exceeds 50 per cent of all random counts in accuracy.

The chamber method shows a deviation from the mean of 4.6 per cent for nearly 200 cells counted and thus is just within the value of S.D. for 200 cells by the random method, i.e., it is as good as about 68.23 per cent of all random counts of 200 cells.

Close agreement between median and mean again indicates that the mean is probably near the true value.

Random count No. 10, made at the margin of the smear, does not indicate that large cells tend to accumulate near the margins. This is in accordance with the finding in blood sample A.

Tables VIII, XI, and X show data obtained and calculated exactly as for Tables V, VI, and VII, except that a third blood sample (sample C) was used. Random count No. 10 was made at the margin of the smear.

The data contained in Tables VIII, IX, and X show that in this case the range, standard deviation, and probable error again decrease as the number of

TABLE VIII  
DATA OBTAINED FROM BLOOD SAMPLE C

NO.	NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHO-CYTE %	LARGE LYMPHO-CYTE %	MONO-CYTE %	EOSINO-PHILE %	BASO-PHILE %
1	100	Random	42.0	50.0	7.0	1.0	0.0	0.0
2	100	Random	39.0	57.0	4.0	0.0	0.0	0.0
3	100	Random	51.0	45.0	4.0	0.0	0.0	0.0
4	100	Random	53.0	44.0	2.0	1.0	0.0	0.0
5	100	Random	48.0	49.0	2.0	1.0	0.0	0.0
6	100	Random	51.0	45.0	3.0	1.0	0.0	0.0
7	100	Random	47.0	52.0	1.0	0.0	0.0	0.0
8	100	Random	48.0	45.0	6.0	1.0	0.0	0.0
9	100	Random	47.0	51.0	2.0	0.0	0.0	0.0
10	100	Random	55.0	42.0	3.0	0.0	0.0	0.0
11	100	Four-field	43.0	53.0	2.0	2.0	0.0	0.0
12	200	Four-field	51.0	48.0	1.0	0.0	0.0	0.0
13	122	Chamber	54.0	40.5	5.1	0.0	0.0	0.0
14	126	Chamber	52.0	46.2	1.5	0.0	0.0	0.0

TABLE IX  
COMPARISON OF DATA FROM BLOOD SAMPLE C

NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHO-CYTE %	LARGE LYMPHO-CYTE %	MONO-CYTE %	EOSINO-PHILE %	BASO-PHILE %
1000	Random average	48.1	48.0	3.4	0.5	0.0	0.0
1000	Random median	48.0	47.0	3.0	0.5	0.0	0.0
100	Four-field	43.0	53.0	2.0	2.0	0.0	0.0
200	Four-field	51.0	48.0	1.0	0.0	0.0	0.0
300	Four-field	48.3	49.7	1.3	0.0	0.0	0.0
248	Chamber	53.0	43.4	3.3	0.0	0.0	0.0

TABLE X  
STATISTICAL SUMMARY OF RANDOM COUNTS FROM BLOOD SAMPLE C

NO. OF CELLS IN COMBINATION	NEUTROPHILES			SMALL LYMPHOCYTES			LARGE LYMPHOCYTES		
	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.
100	13.0	4.6	3.1	15.0	4.4	2.9	6.0	1.8	1.2
200	12.5	3.0	2.0	11.5	2.9	2.0	5.0	1.2	1.0
300	10.3	2.3	1.6	9.6	2.2	1.5	4.0	0.9	0.6

cells counted increases. Likewise, it is necessary to count 300 cells in order to approximately halve the value of S.D. obtained for 100 cell counts by the random method.

The deviation of the 100 cell count for the four-field method is 5.1 for neutrophiles and a deviation this great should occur in only about 27 per cent of random 100 cell counts.

In the 200 cell count by the four-field method, neutrophiles deviate 2.9 from the mean, a deviation which should occur in only about 34.5 per cent of the random 200 cell counts.

In the chamber counts, the average value shows a deviation of 4.9 for neutrophiles from the mean, and only about 10 per cent of 200 cell counts by the random method should show this great a deviation.

TABLE XI  
DATA OBTAINED FROM BLOOD SAMPLE D

NO.	NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHO-CYTE %	LARGE LYMPHO-CYTE %	MONO-CYTE %	EOSINO-PHILE %	BASO-PHILE %
1	100	Random	63.0	28.0	5.0	3.0	1.0	0.0
2	100	Random	59.0	34.0	3.0	3.0	1.0	0.0
3	100	Random	66.0	31.0	3.0	0.0	0.0	0.0
4	100	Random	60.0	32.0	8.0	0.0	0.0	0.0
5	100	Random	61.0	32.0	5.0	2.0	0.0	0.0
6	100	Random	58.0	37.0	4.0	1.0	0.0	0.0
7	100	Random	55.0	43.0	1.0	1.0	0.0	0.0
8	100	Random	56.0	41.0	1.0	2.0	0.0	0.0
9	100	Random	50.0	44.0	4.0	1.0	1.0	0.0
10	100	Random	52.0	41.0	3.0	3.0	0.0	0.0
11	100	Four-field	65.0	32.0	2.0	1.0	0.0	0.0
12	200	Four-field	58.5	38.5	1.5	1.5	0.0	0.0
13	195	Chamber	58.3	38.5	3.1	0.0	0.0	0.0
14	202	Chamber	64.4	34.9	0.8	0.0	0.0	0.0

TABLE XII  
COMPARISON OF DATA FROM BLOOD SAMPLE D

NO. OF CELLS	METHOD	NEUTROPHILE %	SMALL LYMPHO-CYTE %	LARGE LYMPHO-CYTE %	MONO-CYTE %	EOSINO-PHILE %	BASO-PHILE %
1,000	Random average	58.1	36.3	3.7	1.6	0.3	0.0
1,000	Random median	58.5	35.6	3.5	1.5	0.0	0.0
100	Four-field	65.0	32.0	2.0	1.0	0.0	0.0
200	Four-field	58.5	38.5	1.5	1.5	0.0	0.0
300	Four-field	60.7	35.3	1.7	1.7	0.0	0.0
397	Chamber	61.3	36.7	1.9	0.0	0.0	0.0

TABLE XIII  
STATISTICAL SUMMARY OF RANDOM COUNTS FROM BLOOD SAMPLE D

NO. OF CELLS IN COMBINATION	NEUTROPHILES			SMALL LYMPHOCYTES			LARGE LYMPHOCYTES		
	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.	RANGE %	S.D.	P.E.
100	16.0	4.5	3.1	16.0	5.4	3.6	7.0	2.0	1.3
200	13.0	3.1	2.1	14.0	3.6	2.4	5.5	1.3	0.9
300	10.6	2.3	1.6	12.4	2.7	1.8	4.3	1.0	0.7

The close agreement between median and mean would indicate that the arithmetic mean closely approaches the true value.

In this instance, a random count near the margin of the smear indicates the accumulation of neutrophiles near the margin.

Tables XI, XII, and XIII show data obtained and calculated exactly as for Tables V, VI, and VII, except that a different blood sample (sample D) was used. Random counts Nos. 2 and 3 were made at the margin of the smear.

The data contained in Tables XI, XII, and XIII show that the range, standard deviation, and probable error vary inversely with the number of cells counted for random counts. In order to approximately halve the value of P.E. for 100 cell counts, it is necessary to count 300 cells.



The 100 cell four-field count for neutrophiles deviates 6.9 per cent, and the 200 cell count by this method deviates 0.4 per cent from the mean. Only 13.78 per cent of random 100 cell counts should show this great a deviation (6.9 per cent), while the 200 cell count is well within the value of P.E. for neutrophiles.

The chamber method shows a deviation of 3.2 per cent for neutrophiles. If 300 cells had been counted by this method, only about 18 per cent of all random counts should have exceeded this error. Since nearly 400 cells were counted, we should expect even less accuracy as compared with random 400 cell counts.

Close agreement of the arithmetic mean and the median again indicates that the arithmetic mean closely approximates the true value.

Random counts No. 2 and No. 3 in Table XI do not show accumulation of large cells at the margin of the smear in both instances.

#### DISCUSSION OF RESULTS

If the neutrophile values of the first 700 cells from samples A, B, C, and D are averaged and compared with the median, the average (arithmetic mean) of 1,000 cells, and the total white blood cell count, the data shown in Table XIV are obtained.

TABLE XIV

SAMPLE	MEAN 1,000 CELLS	DIFFERENCE FROM MEDIAN	MEAN 700 CELLS	DIFFERENCE FROM MEDIAN	TOTAL LEUCO- CYTE COUNT PER C.MM.
A					
B	51.9	1.1	58.8	1.2	6,825
C	48.1	0.1	55.1	2.1	9,450
D	58.1	0.4	47.3	0.7	6,200
			60.4	2.3	9,925

From these data it would seem that if we assume 1,000 cell values to represent the true values, the counting of 700 cells is not sufficient for absolute accuracy. It is also evident that the higher the total count, the greater the deviation of values based on enumeration of 700 cells.

No agreement is evident between samples as to the types of cells found in preponderance near the margins of the smear. Counts made near the margins do seem to deviate to a greater degree, however, than those nearer the center of the smear.

Ratios of the deviation of four-field meander counts to the probable errors or standard deviations are used to calculate the probability of occurrence of a similar deviation in random counts of the same number of cells.<sup>6</sup> The probable percentage of such deviations in random counts are summarized in Table XV.

It is apparent in each case that four-field counts of 100 cells are not nearly so accurate as random counts. The average of column 3 above, i.e., about 15 per cent, would indicate that about 85 per cent of all random counts of 100 cells should give better values for neutrophiles than four-field counts.

In four-field counts of 200 cells the accuracy is much greater and averages about 58 per cent, thus indicating that only about 42 per cent of random counts of 200 cells exceed this technique in accuracy of the neutrophile count. The

wide divergence of samples C and D, however, indicate that this statement should be verified by further data.

In four-field counts of 300 cells, about 56 per cent of random counts should show greater accuracy and, moreover, the wide divergence of results in the four-field method both with each other and with the values from 200 cell counts would indicate that the sampling is far from satisfactory.

TABLE XV

SAMPLE	100 CELL FOUR-FIELD DEVIATION	APPROX. % OF RANDOM 100 CELL COUNTS EXPECTED TO SHOW THIS GREAT A DEVIATION	200 CELL FOUR-FIELD DEVIATION	APPROX. % OF RANDOM 200 CELL COUNTS EXPECTED TO SHOW THIS GREAT A DEVIATION	300 CELL FOUR-FIELD DEVIATION	APPROX. % OF RANDOM 300 CELL COUNTS EXPECTED TO SHOW THIS GREAT A DEVIATION
A	5.2	9	1.2	50	2.5	10
B	11.1	11	2.9	50	1.8	38
C	5.1	27	2.9	31	0.2	100
D	6.9	14	0.4	99	2.6	28

Thus, lack of sufficient data prevents the drawing of final conclusions as to the reliability of 200 and 300 cell counts by the four-field method, but it is probable that the data shown indicate definitely the unreliability of the method.

Deviations of counting chamber values from the true mean are so great as to make this technique as outlined in this paper valueless. However, it is possible that a similar technique, using greater magnification, would lessen the deviation.

A comparison of standard deviations for neutrophils for 100, 200, and 300 cell counts and total leucocyte count is shown in Table XVI

TABLE XVI

SAMPLE	LEUCOCYTES PER C.MM.	S.D. 100 CELLS	S.D. 200 CELLS	S.D. 300 CELLS
A	6,825	3.0	1.9	1.4
B	9,540	6.9	4.6	3.5
C	6,200	4.6	3.0	2.3
D	9,925	4.5	3.1	2.3

Apparently there is no connection between total leucocyte count and the standard deviation in these cases. Thus, it would seem that 100 cell counts would be as accurate for high total leucocyte counts as for low. Here, again, however, insufficient data do not permit definite conclusions to be drawn.

From the data shown, it would seem that 300 cells should be counted in smears prepared as outlined in order to obtain a reasonable degree of accuracy.

## CONCLUSIONS

1. In smears prepared as outlined, the enumeration of 700 to 1,000 cells gives nearly true values for the differential count by the random method.
2. Four-field meander counting is inferior to random counting on the basis of 100 cell counts and does not seem to be a satisfactory sampling procedure for 200 or 300 cell counts.

3. Differential counting from the counting chamber by the technique outlined seems to be an inaccurate procedure.

4. In order to reduce deviations to reasonable minima, 300 cells by the random method should be counted.

5. Further work to determine the effect of the total leucocyte count upon the probable error and standard deviation and the reliability of the 200 and 300 cell four-field method is desirable.

#### SUMMARY

A statistical evaluation of certain techniques of differential leucocyte enumeration has been made. Random counting on uniform smears is shown to be at least as accurate as four-field counts of 200 and 300 cells, and more accurate than 100 cell counts by the latter method.

It is shown that reasonable accuracy can be obtained by random counting of 300 cells in most instances and that the counting chamber technique of differential enumeration as described is valueless.

The author extends thanks to J. Willey Morrison, technician, Southwestern State College, Department of Student Health, for the preparation and enumeration of the blood samples upon which this paper is based.

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## MODIFIED TECHNIQUE FOR STAINING CAPSULES OF *HEMOPHILUS PERTUSSIS*\*

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IN 1905, Wherry<sup>1</sup> described the use of Wright's stain in demonstrating a previously undescribed capsule surrounding *Pasteurella pestis* and *Pasteurella borisepctica*. Smears made from young cultures of these organisms showed distinct capsules when stained by his method, but direct smears from animal lesions failed to reveal this structure. Churchman and Emenianoff,<sup>2</sup> evidently unaware of the previous work of Wherry on this subject, redescribed the method and its application to the demonstration of capsules and applied this technique to numerous organisms.

In 1931 an attempt<sup>3</sup> was made to apply this method to *H. pertussis*. Small areas in slides so stained showed grayish amorphous substance surrounding the bacterial cell. In an endeavor to bring out this structure, I had recourse to several modifications of the technique and was able to demonstrate a capsule-like substance by the following technique:

The growth of *H. pertussis* on Bordet medium is taken up on a platinum loop or a dry sterile throat swab and smeared in a thin film on the surface of a scrupulously clean slide. No diluent of any sort is used. The films are dried in air, fixed with absolute methyl alcohol for one minute, and the slide then covered with 10 to 20 drops of a staining solution composed of two parts Wright's stain and one part glycerin, freshly made and well mixed. This is allowed to remain for two minutes in contact with the smear, and 20 to 30 drops of distilled water are then added and mixed with the stain by the use of a capillary pipette. The smear is stained for ten to twenty minutes, rinsed, dried, and examined.

Capsules could not be demonstrated when saline solution or water were used as emulsifying agents. The use of unmodified Wright's stain frequently gave good results, but not so consistently as the method described. Giemsa's stain, which contains glycerin and is similar in its composition to the author's modified stain, failed to demonstrate capsules. Freshly mixed glycerin and Wright's stain appeared to be indispensable in bringing out this structure.

The best results were obtained by using very young cultures of smooth organisms (phase 1), although dissociated organisms (phases 3 and 4) also possess capsules. As with all capsule stains, the results on any given slide were not uniform throughout, and only occasional areas in the stained preparation showed the capsule to good advantage. Usually where the smear was thinnest and the polychrome effect of the stain most noticeable, excellent capsules were seen. Promising areas, pink in color under the low-power objective, were first found, and these areas were examined with an oil-immersion objective.

\*From the Department of Medicine, University of Virginia, Charlottesville.  
Received for publication, May 19, 1939.

The foregoing technique has been used by numerous workers, and its effectiveness in demonstrating capsules of *H. pertussis* has been confirmed by Shibley and Hoelscher,<sup>4</sup> Evans and Maitland,<sup>5</sup> and others.<sup>6</sup>

I have never been completely satisfied with this stain and have recently re-examined the problem with the idea in view of producing more uniform results. Smith's<sup>7</sup> stain has given very excellent results in demonstrating capsules of practically all organisms in which this structure can be shown by other methods. This stain, however, failed to show any capsular material surrounding *H. pertussis*. It was thought, however, that a combination of the technique of fixation used by Smith and my modification of the Wright stain might be productive of results. This was found to be true, and a slightly modified stain, which has given excellent results, is here described.

Dry smears are made on the slide as in the previous method. These are allowed to dry in air and then covered with a 5 per cent aqueous solution of phosphomolybdic acid. This is allowed to remain in contact with the smear for thirty seconds and washed off completely in running water and then washed with methyl alcohol. The staining procedure is thereafter the same as in the first method.

By the use of this stain it was possible to demonstrate capsular material in much more extensive areas than with the first method. Due to the acid fixation, the tinctorial results were somewhat different. Capsular material was light sky-blue color, and the organisms themselves were dark blue when crowded, and magenta to red when lying in thin areas on the slide. Only in the latter situation were capsules stained to best advantage. Both Wright's stain and Giemsa's stain could frequently be employed with good results following this fixation, but the best results were still obtained by the use of the technique previously given.

During the course of recent experiments undertaken in the hope of increasing the virulence of *H. pertussis* for mice, I had occasion to utilize a Bordet-Gengou base medium with additions of mucin. Several strains of *H. pertussis* grown on this medium showed considerably increased virulence for mice when instilled into the nares according to the technique of Burnet and Timmins<sup>8</sup> or into the larynx by the method I have recently described.<sup>9</sup>

It was also discovered that *H. pertussis*, when grown on this medium, showed capsules which were larger and more constant throughout all fields than with any cultures which had been examined previously.

The technique of preparing this medium is as follows:

To the Bordet-Gengou medium, as modified by Kendrick, Miller, and Lawson,<sup>10</sup> mucin\* is added to make from 5 per cent to 8 per cent of the final volume. The mucin is best dissolved by grinding the granular mucin to a fine powder in a mortar and gradually adding distilled water, constantly grinding the resulting sticky mixture until it reaches a homogeneous state and the consistency of thick cream. The distilled water used in dissolving the mucin is subtracted from that used in the agar-salt mixture, and the agar and salt are dissolved in the remainder.

\*Wilson Laboratories Granular Mucin Type 1701-W was used throughout these experiments.

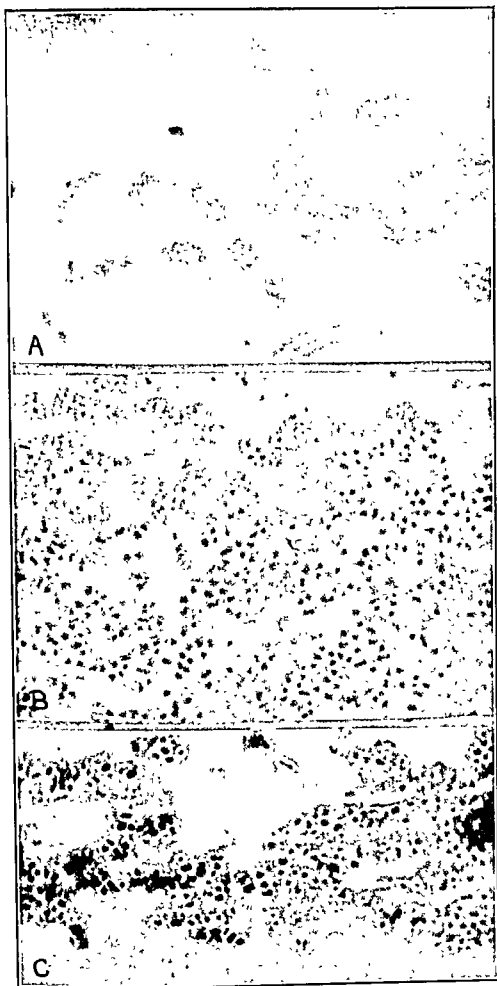


Fig. 1.—A. Capsule stain, original method, rough culture (phase 4) ( $\times 3500$ ). B. Capsule stain, first modification, smooth culture (phase 1) ( $\times 2400$ ). C. Capsule stain, second modification, mucin grown smooth culture (phase 1) ( $\times 2400$ ).

The two solutions are then mixed and added to the potato-glycerin extract. The resulting medium is dispensed in flasks in convenient volume and sterilized in the autoclave for twenty minutes at 15 pounds pressure. To the above base, when cooled to 45° C., sterile blood is added to make a 15 per cent to 20 per cent concentration. Rabbit blood was used in the experiments outlined, but other blood—sheep, horse, and human—has also been used successfully. Plates are poured and allowed to solidify.

The final consistency of this medium is so soft that it cannot be inoculated satisfactorily with a platinum loop. A sterile throat swab used instead of the loop, seeded with *H. pertussis* and drawn very lightly over the surface of the medium, inoculates the plate satisfactorily. A similar sterile swab is used to transfer the growth obtained after from twenty-four to forty-eight hours' incubation at 37° C. to clean dry slides, making thin smears which are air dried, fixed in absolute methyl alcohol, and then stained with my glycerin-Wright stain mixture.

The results of this technique are so far superior to those obtained by the two methods previously outlined that the method is recommended to all investigators who are engaged in research on *H. pertussis*.

#### SUMMARY

Two modifications of my original stain for capsules of *H. pertussis* are described, one relying on fixation of the smear with phosphomolybdic acid prior to the use of the stain; the second dependent upon the growth of *H. pertussis* on a Bordet-Gengou base containing mucin. This latter procedure seems to increase the virulence of *H. pertussis* for mice as well as furnishing a growth showing capsules to their best advantage.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**BILE, Bilirubin and Urobilin Content of, Obtained by Duodenal Drainage, Royer, M.** Arch. Int. Med. 64: 445, 1939.

The concentrations of bilirubin and urobilin are higher in B bile than in A bile.

The increase in concentration of urobilin is normally lower than that of the bilirubin. This is due to the fact that only a small amount of bilirubin is absorbed by the wall of the gall bladder, while urobilin is absorbed in surprisingly large amounts.

In a certain number of cases of cholecystitis (41.4 per cent of author's cases) the production of urobilin in the gall bladder can be observed. This fact explains why the ratio of increase of bilirubin to increase of urobilin is lower than the normal ratio. Data in this regard can be of use in making a differential diagnosis of inflammation of the gall bladder.

**SILICOSIS, Dyspnea of, What Causes It? Cole, L. G., and Cole, W. G.** J. A. M. A. 113: 1216, 1939.

Dyspnea is the outstanding symptom of silicosis, but it has not been accounted for by hitherto recognized pathologic changes. It is frequently absent in cases of marked nodulation and is, on the other hand, present in cases of indefinite nodulation.

A consideration of silicosis under the three types previously recognized—peribronchial, nodular, and acute—aids in the comprehension of this problem. The acute manifestation, as observed in the Gauley Bridge cases, gave the key to this silicotic problem.

Large sections from the hilus to the pleura stained with Mas-on's trichrome stain were most valuable in enabling us to make the observations reported. Black and white photomicrographs were useless, and hematoxylin and eosin stain was also unsatisfactory.

Large microscopic sections of silicotic lungs show three general manifestations of morbid changes: (1) avascular areas, (2) overvascular areas, and (3) a region of invasion.

The region of invasion, where collagen constricts the capillaries, is the most important region for intensive study. In the avascular portion, capillary occlusion by external pressure causes a traffic jam of red blood cells, with a damming back of the blood into the arterioles and a lack of drainage of the veins. The blood in the large vessels in the avascular areas disintegrates. Overvascular areas, where there is immense dilatation of the capillaries, and engorgement of the veins and arteries with viable blood cells, seem to compensate for avascular regions in different portions of the section.

Theoretically dyspnea has been explained by two pathologic factors: (1) nodulation and (2) alveolar consolidation. To these we add a third, capillary obstruction and dilatation.

From a study of microscopic sections we have made certain deductions which constitute our conception of the life history of silicosis at least as it occurs in some groups of cases. We believe that the dyspnea of silicosis is due to capillary occlusion in large localized regions of the lung, with compensating capillary dilatation in other regions.

Most diseases exhibit morbid changes which can be recognized in relatively small sections; for example, certain types of neoplasm can be determined by small groups of characteristic cells, but in silicosis there is a vast variety of morbid changes in various portions of the lung. Different varieties of dust result in different types of morbid change in varying regions of the lung.



**TISSUES, Fixing and Staining Methods for Lead and Copper in, Mallory, F. B., and Parker, F., Jr. Am. J. Path. 15: 519, 1939.**

*Hematoxylin Stain:* Hematoxylin and its ripened derivative, hematein, unite with a number of metals to form colored compounds, some of which have been found very useful as stains for nuclei and other tissue elements. As a rule, hematein is required to make the stain effective. For this reason, an alum hematoxylin solution must be ripened by the aid of light, heat, or oxidizing reagent. For staining lead, hematoxylin itself is essential, and ripening of the staining solution must be prevented as far as possible. On this account a solution in dibasic potassium phosphate has been found most useful.

*Fixation:* Tissues to be examined for lead must be fixed in 95 per cent or absolute alcohol. Formalin is worthless and, therefore, tissues fixed in this reagent in the past are useless.

*Method of Staining:* Stain celloidin sections in the following solution in the paraffin oven (about 54° C.) for 2 to 3 hours, rarely longer. Paraffin sections are loosened from the slide.

Dissolve 5 to 10 mg. (but not more) of hematoxylin in a few drops of absolute or 95 per cent alcohol, and add 10 c.c. of a freshly filtered 2 per cent aqueous solution of dibasic potassium phosphate. After staining, wash the sections in several changes of tap water for 10 minutes to 1 hour, dehydrate in 95 per cent alcohol, clear in terpineol, and mount in terpineol balsam.

*Results:* By this method lead is stained a light to a dark grayish blue, and the nuclei (which owe their staining properties to the presence of metals) take a deep blue color.

Copper is fixed well by both alcohol (95 per cent or absolute) and neutral formalin, and is stained intensely blue by hematoxylin and by hematein. The simplest method is to use the same solution recommended for staining lead. The iron so commonly associated with copper stains black after alcohol fixation, but light to dark brown after formalin fixation.

**GLUCOSE TOLERANCE, The One-Hour Two-Dose Dextrose Tolerance Test (Exton-Rose Procedure), Matthews, M. W., Magath, T. B., and Berkson, J. J. A. M. A. 113: 1531, 1939.**

Advancing age produces a progressive elevation of the blood sugar level at every phase of the blood sugar time curve obtained with the dextrose tolerance test of Exton and Rose. The degree of this, however, is insufficient to invalidate the following conclusions:

A fasting blood sugar that exceeds 120 mg. per hundred cubic centimeters of blood is diagnostic of diabetes. This value, however, was exceeded by only 21 per cent of the persons with diabetes in this series of cases in which diabetes was minimal. In contrast, no person in whom the carbohydrate tolerance was considered normal had a fasting blood sugar that exceeded 110 mg.

According to our experience, the most effective criterion with the Exton-Rose procedure for differentiating persons suffering from diabetes and normal persons, is the hour value of the blood sugar. If 158 mg. per hundred cubic centimeters of blood are taken as the critical level so that individuals showing a blood sugar reading below this level at the hour are designated nondiabetic, and individuals with readings at or above this value are designated presumptively diabetic, a high percentage of correct diagnoses can be expected. As far as the observations in this series are concerned, all individuals with values at the hour less than 154 mg. were found to be normal, and all individuals with values at the hour of 180 mg. or more were found to be diabetic. Hence these two groupings are most definite. Individuals with values at the hour between 158 and 179 mg., inclusive, constituted only a small fraction of our cases (6, or 5.1 per cent, of 117 patients considered to be nondiabetic, and 19, or 7.7 per cent, of 247 patients considered to have latent or mildest diabetes). The number of cases with a doubtful laboratory diagnosis was smaller by this criterion than by any other criterion applied to the results of the Exton-Rose test. It also was smaller than that obtained by any criteria applied to the interpretation of other oral tests for dextrose tolerance with which we have had experience.

**TUBERCLE BACILLI**, Clinical Significance of, in Urine, Dukes, C. E. Brit. M. J. 21: 799, 1939.

Tuberculous bacilluria is the result of an occult or subclinical tuberculous infection of the kidney, and may occur in many different forms of extrarenal tuberculosis, especially in disease of bones and joints. Tubercle bacilli are present in the urine in small numbers only, and are rarely found by microscopical examination. Guinea pig tests are necessary to demonstrate tuberculous bacilluria. The excretion of tubercle bacilli is intermittent. The urine is generally clear in appearance, though containing an excess of leucocytes. The excretion of tubercle bacilli may cease when the patient's general health improves.

In clinical urinary tuberculosis the urine contains large numbers of pus cells, and these can generally be found in stained films. Guinea pig tests and cultures are rarely necessary. The excretion of tubercle bacilli is constant, and will continue as long as an open tuberculous lesion can discharge freely into the urinary tract. In clinical urinary tuberculosis the urine always contains a few pus cells, and often the pyuria is sufficient to cause a definite turbidity. These points enable a distinction to be made between results of laboratory tests in tuberculous bacilluria and in clinical urinary tuberculosis.

**SPUTUM**, Technique of Examination of, Pottenger, J. E. Am. Rev. Tuberc. 40: 581, 1939.

A report on the examination of successive twenty-four-hour specimens of sputum by the dilution-flotation picric acid method and guinea pig inoculation records that of a total of 171 specimens; 57 were positive to guinea pig; 18 were positive in a ten-minute search after treatment by dilution-flotation; and 39 were positive after prolonged search (thirty to ninety minutes).

The dilution-flotation technique will detect tubercle bacilli in a ten-minute search if present to the extent of about 175 per c.c. of natural specimen. Below this number the guinea pig is advisable for diagnostic purposes.

Guinea pig inoculation, if negative, is wholly inadequate for diagnosis, if only a twenty-four-hour inoculum is used. Only 25.5 per cent of the specimens from a group of 8 patients in whom a definite diagnosis had been made were positive in guinea pigs. A statistical study shows that a three-day inoculum would be positive in 51.6 per cent; and a five-day, seven-day, nine-day, and fifteen-day inoculum in 66.1, 74.2, 78.6, and 86 per cent, respectively.

**PROTHROMBIN**, Study of the Quick Method for Quantitative Determination With Suggested Modifications, Pohle, F. J., and Stewart, J. K.: Am. J. M. Sc. 198: 622, 1939.

The Quick method for the quantitative determination of plasma prothrombin contains variables which significantly influence the results of the test.

Calcium is an important variable in the Quick prothrombin method. The optimal amount of calcium necessary for recalcification must be determined in each instance to assure a minimal coagulation time (prothrombin time). Studies on the plasma of 85 normal individuals indicate that with optimal recalcification the normal prothrombin time is 10 seconds.

The optimal calcium concentration required for the determination of the Quick prothrombin time is similar to normal blood serum calcium values.

Slight variations in the oxalate concentration of the plasma do not influence prothrombin determinations if the optimal amount of calcium required is determined in each case.

The presence of a gross lipemia significantly shortens the prothrombin time. It is suggested that in order to avoid this variable, determinations should be done only on clear plasma.

Observations on 46 individuals, with liver or biliary tract disease, indicate that the modified Quick prothrombin method measures a deficiency which frequently exists in these cases, and which is apparently responsible for the bleeding tendency.

**MYELOMATOSIS**, Diagnosing, by Complement Fixation, Jersild, M. J. A. M. A. 113: 1119, 1939.

Of 27 patients with anticomplementary serum, 13 definitely and 5 probably had myelomatosis. The reaction occurs only after the serum is heated to from 56° to 60° C., and a considerable increase in globulin is often found; the coagulation temperature of the serum is often low.

**FLUIDS**, Carcinoma Cells in Thoracic and Abdominal, Schlesinger, M. J. Arch. Path. 28: 283, 1939.

The diagnosis of carcinoma can be made readily on thoracic and ascitic fluids. Consistent, reliable results can be obtained best by the technique of centrifugation of the fluid, and fixation, embedding, and sectioning of the sediment. The method of utilizing stained or unstained smears was found to be unreliable.

A definite positive diagnosis of carcinoma should be made on such sections only after finding groups of definite polygonal cells showing polarity, sharply distinct cell walls, and acinar or pseudoacinar formation.

Such readily recognizable microscopic bits of tumor tissue are contained in about 60 per cent of the fluids accumulating in the cavities of the body as the result of carcinoma.

The cells of fluids accumulating in the cavities of the body from other causes never assume such a histoid appearance but may otherwise resemble carcinoma cells. If strict adherence to the aforementioned proper, rigid criteria is maintained, a false diagnosis of carcinoma will never be made on such a nonneoplastic fluid.

The author's method follows: The technique followed is extremely simple. The fluid is centrifuged at high speed until the supernatant liquid is clear. This is most conveniently accomplished by centrifuging repeatedly in the same 50 c.c. round bottom centrifuge tube, pouring off the cleared fluid and replacing it by fresh fluid to be centrifuged. Sufficient sediment is thus finally obtained as one mass at the bottom of the centrifuge tube. Even if the fluid has not been collected aseptically, its preservation in the icebox for twenty-four or thirty-six hours before centrifuging permits little effect on the neoplastic cells. Centrifuging for a considerable period at the highest speed obtainable does not damage the cells.

The sedimented mass obtained is then prepared for fixation. It is first transferred to a piece of filter paper as a small compact pile. This transfer is made either with a small spatula, with the point of a scalpel, or with a small platinum loop, depending on the amount and consistency of the sediment. The sediment obtained is seldom insufficient for such transfer, especially the sediment from fluids containing neoplastic cells. Sometimes a fibrin clot forms in the fluid and a voluminous jelly-like precipitate, which comes out of the tube en masse, is obtained after centrifuging. In this clot are enmeshed all the cells of the fluid. Before fixing, some of the fluid that is also enmeshed in the clot is extracted by gently rolling the clot around on dry filter paper. The sediment or clot is fixed in at least 10 volumes of a neutral 1:10 dilution of solution of formaldehyde U.S.P. for eighteen to twenty-four hours, embedded in paraffin, sectioned at 8 to 10 microns, and stained with hematoxylin and eosin.

**LEUCOCYTES**, Chemotropism of Human Eosinophilic Polymorphonuclear, Ingraham, E. S. and Wartman, W. B. Arch. Path. 28: 318, 1939.

The chemotropism of human polymorphonuclear eosinophiles was studied *in vitro*. The cells were obtained from a patient with eosinophilic myelogenous leucemia and from one with dermatitis herpetiformis. The chemotactic reaction of eosinophiles to bacteria was found to be as strong as that of normal neutrophilic leucocytes. Eosinophiles were attacked less strongly by material from an animal parasite, *T. spiralis*, than by Witte's peptone.

**GROSS PATHOLOGIC SPECIMENS, Solid Carbon Dioxide in Preparation of, Bauer, J. T.**  
*Arch. Path.* 28: 396, 1939.

A fiber packing box or container large enough to hold the part to be frozen is obtained and lined with an old woolen blanket and a few newspapers. On this the specimen is placed, and then small pieces of solid carbon dioxide (5 to 25 cm. in diameter) are placed about the specimen until it is entirely surrounded. (Heavy gloves should be worn to prevent severe frostbite while cracking the solid carbon dioxide and packing it about the specimen and while handling the specimen after it is frozen.) The newspapers and blanket are then tightly wrapped about the solid carbon dioxide.

Depending on the temperature of the room and the size of the specimen, a block or two of solid carbon dioxide [50 to 100 pounds (22.7 to 45.4 kg.)] will suffice to freeze an arm or a leg solid within twenty-four to thirty-six hours. At the end of this time gross sections can be sawed in any plane through skin, subcutaneous fat, muscles, blood vessels, and bone, without tearing or distorting the tissue. (Author used a carpenter's crosscut saw with 8 or 9 teeth per inch.) The sawdust that adheres to the cut surface of the specimen is quite dry at first and can be scraped away with the edge of a knife.

If the cut surface is then gently and quickly washed with cold water, the water will be frozen into a thin smooth transparent layer of ice, which readily permits photography with few or no dazzling high lights or reflections. Color photographs of such specimens are especially pleasing, as the natural colors are preserved.

Blocks can then be taken from the frozen specimen with a saw and chisel, and placed in the usual histologic fixatives for the preparation of material for microscopic study. No detrimental effect other than slight shrinkage has been noted in the microscopic sections of tissue frozen by this means. Although chemical analyses of tissue frozen by solid carbon dioxide have not been made in the author's laboratory, they should be possible, as rapid freezing retards most chemical changes.

Modifications of the method outlined have been suggested but not tried. These include injection of the vascular bed of the specimen with fixatives before freezing and use of a band saw or other kinds of power saws instead of a hand saw.

The cost is little more than that of the solid carbon dioxide, which in the author's experience was between \$3.00 and \$3.50 per specimen.

**SULPHANILAMIDE, Effect of, on Spermatogenesis in Man, Heckel, N. J., and Hori, C. G.**  
*Am. J. M. Sc.* 198: 347, 1939.

In contrast to other reports, there were no noteworthy effects in 11 patients upon the total number or percentage of live spermatozoa from the use of sulfanilamide.

Such variations, as shown in the total spermatozoa counts during and after treatment, are no greater than the variations which occurred before the drug was given.

**POLIOMYELITIS, Study of Heterologous Antibodies in, Burnet, F. M., Freeman, M., Jackson, A. V., and Lush, D.** *M. J. Australia* 11: 198, 1939.

Seventy specimens of serum from patients with poliomyelitis have been examined for their content of heterologous antibodies against herpes simplex and swine influenza viruses and for diphtheria and staphylococcal antitoxins. The results have been analyzed according to age and social environment and compared with those from control groups.

There is no evidence that any of the heterologous antibodies in these specimens of serum are in any different proportion from those which would be found in a comparable group of normal specimens.

Herpes simplex antibody and diphtheria antitoxin are both much more frequently found in children from crowded industrial suburbs than in those from better class areas. In the industrial areas herpes infection is contracted before the age of two years, while diphtheria antitoxin appears, as a rule, only after the child has begun to attend school.

There is no association between the frequency of swine influenza antibody or staphylococcal antitoxin in serum and the nature of the social environment.

There is a significant correlation between the presence of poliomyelitis antibody and diphtheria antitoxin in young children from industrial suburbs; this suggests that the two antibodies are acquired at about the same age and under similar environmental conditions.

# REVIEW

## The Story of Surgery\*

Some years ago Hugh Walpole divided a little essay upon "Reading" into three main headings: "Reading for Fun," "Reading for Love," and "Reading for Education."

Readers of the *Story of Surgery* will have difficulty determining under which of the aforementioned headings it best falls and will probably end by concluding that it embodies a little of all three. The lay reader will read it "for fun" and "for education"; the doctor and those with whom he properly has kin will read it "for fun" also, but likewise "for love"; for it is, indeed, a colorful, well-conceived and well-written tale of the past and present and, withal, casts a questing eye into the future.

This reviewer agrees entirely with Grogarty: "This is the best book on surgery I have ever read." Lest any one be led by this to conclude hastily that it is a textbook, a dry tome of technique, let it be added that it is also a mine of folklore and superstition, as well as a treasure house of anecdote and characterization.

"Harvey Graham" is a pseudonym, though it is not clear why the author should thus "hide his light under a bushel," for he has written exceedingly well, with a lively, scholarly, and yet, at times, witty style.

Medicine in all its phases began in superstition, ignorance, mystery, and magic. This the author has well limned in his opening chapter, "The Dawn of Surgery," with its picture of primitive surgery among neolithic men. The picture is drawn with swift, sure strokes, and stands out like a vignette. You sense at once that the author not only has imagination but also has skill to transfer it to his pages.

Space does not permit an extensive review, but a few of the chapter headings may be quoted just to whet the appetite, such as: "The Quacks and the Brothers," "Corpses, Curiosities and Quacks," "Barbers and Chirurgeons," "Surgeons Become Gentlemen," "Struggles for Corpses," and "The Black Death." There are, in all, twenty-five chapters which, beginning in the murky mists of primordial ages, end with "Today and Tomorrow."

This is a book that can—and will be—read from start to finish without a stop. But it is also a book that can be reread and browsed in. This is the mark of a book worth while.

It is a little unfortunate that the author chose a rather prosaic title, for it does not begin to convey the intriguing interest of the book.

There are numerous excellent illustrations and a comprehensive index. Without doubt, the purchase of this book is an investment rather than an expenditure.

\*The Story of Surgery. By Harvey Graham. With a Foreword by Oliver St. John Grogarty. Cloth, 425 pages, 23 illustrations, \$3.75. Doubleday, Doran & Co., Inc., New York, N. Y.

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## CLINICAL AND EXPERIMENTAL

### THE BLOOD PIGMENTS\*

THE PROPERTIES AND QUANTITATIVE DETERMINATION WITH SPECIAL REFERENCE  
TO THE SPECTROPHOTOMETRIC METHODS

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WITH the increasing prevalence of aniline-containing drugs and poisons in the past few years, a number of reports concerning the presence of abnormal pigments in the blood have appeared. The constitution of the hemoglobin derivatives has been but recently clarified, and the nomenclature has not as yet been standardized. Methods for the quantitative determination of methemoglobin and sulfhemoglobin have not been adequately reviewed. At present, they are often not specific for the pigment in question, and are sufficiently cumbersome to limit clinical studies to small series of cases. We have reviewed (in this study) the interrelationships and characteristics of blood pigments and presented a critical discussion of existing methods, together with rapid, simple methods, for the spectrophotometric determination of methemoglobin and sulfhemoglobin.

#### NATURE OF THE BLOOD PIGMENTS<sup>1-3</sup>

The prosthetic group of the hemoglobin compounds is an iron protoporphyrin complex termed *heme* by Anson and Mirsky.<sup>†</sup> The reduced, or ferriheme, compound may be reversibly oxidized at the iron atom to ferriheme

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<sup>†</sup>Nomenclature of the Blood Pigments. Pauling and Coryell<sup>2</sup> have recently advanced certain improvements in the nomenclature. The following table is added to simplify the relationships. The terms of Pauling and Coryell are italicized while those of Anson and Mirsky<sup>1</sup> are in parentheses.

*Heme*: A generic term for any iron porphyrin complex.

(hematin).<sup>4</sup> The latter combines with acids to form ferriheme salts (e.g., ferriheme chloride) or with alkalis to form ferriheme hydroxide (alkaline hematin).<sup>5</sup> Ferroheme may combine with certain nitrogenous substances (e.g., proteins, pyridine, cyanides) to form ferrohemochromogens,<sup>6</sup> while ferriheme forms a corresponding series of ferrihemochromogens (parahematin).<sup>5</sup> The combination of ferriheme and native globin has been performed in vitro and results in ferrihemoglobin (methemoglobin).<sup>7</sup> Ferroheme and native globin form ferrohemoglobin (hemoglobin). The oxidation of hemoglobin which results in ferrihemoglobin (methemoglobin) is to be contrasted with the oxygenation of hemoglobin which results in the formation of the addition complex, oxyferrohemoglobin (oxyhemoglobin).

Sulfhemoglobin has never been prepared in a pure state and its constitution is not known.<sup>9</sup> Upon disrupting the molecule, one obtains the same heme and porphyrin as in the case of hemoglobin.<sup>10</sup> Alkaline denaturation experiments show that there is no major change in the globin portion of the molecule.<sup>11</sup> The formation of sulfhemoglobin apparently involves the introduction of one atom of relatively labile sulfur into the hemoglobin structure and also a change in the mode of linkage of the globin to the heme. At the present time, there is no way of converting sulfhemoglobin into either methemoglobin or hemoglobin without first splitting the molecule into its constituent groups.

The relationship between the blood and bile pigments is best summarized by a consideration of the recent work of Lemberg<sup>12</sup> and of Barkan and Schales.<sup>13</sup> Under certain experimental conditions, the partial oxidation of hemoglobin or hemochromogen leads to an opening of the porphyrin structure at the alpha methene bridge without a loss of the central iron atom. To this type of compound Barkan has applied the term pseudohemoglobin, while Lemberg calls them verdohemochromogens. A characteristic of this group is the ease with which the iron may be split off by mild acids to give rise to the bile pigment, biliverdin. Pseudohemoglobin may furnish the link between hemoglobin and the bile pigments. Barkan has suggested that the 4 to 6 per cent of labile iron in the red blood cells<sup>14</sup> may be due, in part, to the presence of pseudohemoglobin.<sup>15</sup>

#### CHEMICAL PROPERTIES

Hemoglobin combines reversibly with oxygen, carbon monoxide, and nitrous oxide.<sup>16</sup> Through the action of strong acids or bases upon hemoglobin, the cor-

*Feroheme* (reduced heme): Ferrous porphyrin complex. Unless specified, the porphyrin is protoporphyrin.

*Ferriheme* (oxidized heme): Ferric porphyrin complex.

*Ferriheme chloride* (hemin): Teichmann's crystals. Ferriheme plus chloride ions. The general term for these salts is acid hematin.

*Ferriheme hydroxide* (alkaline hematin): Ferriheme plus hydroxyl ions.

*Ferrohemochromogen* (hemochromogen): Complex of ferroheme and one or two nitrogenous substances, such as pyridine, nicotine, ammonia, etc.

*Ferrihemochromogen* (parahematin): Also called kathematin. Complex of ferriheme and nitrogenous substances.

*Hemoglobin*: A generic term denoting the complex or an iron porphyrin and native globin.

*Ferrohemoglobin* (reduced hemoglobin or hemoglobin): Complex of ferroheme and native globin.

*Oxyhemoglobin*: The complex of ferrohemoglobin and oxygen.

*Denatured hemoglobin*: The complex of ferriheme and denatured globin.

*Parahemoglobin* (parahematin or kathemoglobin): The complex of ferriheme and denatured globin.

A system for naming the hemochromogen derivatives is given by Drabkin.<sup>8</sup>

responding ferrihemes are formed. Oxidation with hydrogen peroxide in the presence of large amounts of cyanide or coupled oxidation with ascorbic acid results in the formation of pseudohemoglobin.<sup>17</sup> Strong oxidation with a variety of agents changes the valence of the iron to form methemoglobin. Hemoglobin and oxyhemoglobin do not react with cyanides, fluorides, or azides.

Methemoglobin forms a reversible redox equilibrium with hemoglobin. The  $E_0$  at pH 7 is + 150 millivolts.<sup>17</sup> Ozone, iodine, chlorates, ferricyanide, hydroxylamine, hydroquinone, p-amino phenol, formaldehyde, hydrogen peroxide, pyrogallol, permanganate, and certain bacteria (*Streptococcus viridans*, cholera vibrio, pneumococcus, Gärtner's bacilli, and certain nitrosobacilli) can convert hemoglobin to methemoglobin.<sup>18</sup> Methemoglobin forms spontaneously when solutions of purified hemoglobin are exposed to oxygen. The free energy of this reaction is sufficiently great to preclude the spontaneous occurrence of the reverse reaction.<sup>19</sup> However, in vivo or in drawn whole blood, the reverse reaction may take place through the action of reducing substances (glucose, unsaturated fatty acids) which are present.<sup>3</sup> Methemoglobin is reduced to hemoglobin by the action of sodium hydrosulfite, ammonium sulfide, sodium anthrahydroquinone disulfonate, titanous tartrate, and Stokes' reagent. Methemoglobin combines reversibly with cyanides (formation of cyanmethemoglobin),<sup>20</sup> sulfides (sulfmethemoglobin),<sup>21</sup> peroxides,<sup>22</sup> fluorides,<sup>23</sup> and azides.<sup>24</sup> Like the other ferriheme compounds, it may exist as the base (alkaline methemoglobin) or as the salt (acid methemoglobin).<sup>25</sup> It does not combine with oxygen or carbon monoxide.

Sulfhemoglobin is formed by the reaction of hemoglobin with soluble inorganic sulfides and hydrogen peroxide.<sup>11</sup> The reaction of oxyhemoglobin with sulfides is slow but is considerably accelerated by certain reducing agents (phenylhydrazine, hydrazine, hydroxylamine, naphthylamine, phenylenediamine, isatine, paraminophenol, and particularly hydrosulfite).<sup>27</sup> Sulfhemoglobin exists in the oxidized or reduced form.<sup>21</sup> As ordinarily prepared through the action of sulfide and a reducing agent on oxyhemoglobin, the reduced form is obtained. Oxidized sulfhemoglobin may be prepared by the action of ferricyanide on the reduced form. Reduced sulfhemoglobin reacts with carbon monoxide,<sup>27a</sup> but not with oxygen, cyanide, sulfides, ammonia, azide, hydrosulfite, Stokes' reagent, alkalis, or fluorides.<sup>26</sup> Hemochromogen is formed upon the addition of mercuric chloride or strong bases followed by reduction with hydrosulfite.<sup>28</sup>

Hematin is formed through the action of strong acids or bases upon hemoglobin. As has been mentioned previously, it may exist as the base or salt. Hematins combine with cyanides to form cyanhematin and with other nitrogenous compounds to form parahematins.<sup>5</sup> Upon reduction, hematins form unstable ferrohemes which rapidly revert to hematin in the presence of oxygen. Both hematin and cyanhematin form ferrohemochromogens upon the addition of ammonium sulfide.<sup>29</sup> This behavior is to be contrasted with that of methemoglobin or cyanmethemoglobin which slowly revert to reduced hemoglobin under the same conditions. Ferroheme and ferrohemochromogen, particularly in alkaline solutions, combine with carbon monoxide but not with oxygen.<sup>30</sup> Hematin has been reported in the blood plasma and urine of patients with malaria, pernicious anemia, intravascular hemolysis, and sepsis.<sup>31</sup>



A pigment, known as pseudomethemoglobin, may appear in the plasma of patients with blackwater fever or massive intravascular hemolysis.<sup>32, 33</sup> This pigment is identified by the presence of an absorption band between 622 and 624 m $\mu$ , by its reaction with sodium hydrosulfite, and by its failure to react with Stokes' reagent or 10 per cent ammonium sulfide. The composition is not known. Fairley and Bromfield<sup>32</sup> have suggested that pseudomethemoglobin is a combination of hematin with unknown nitrogenous constituent of the plasma, probably serum albumin. The pigment has not been found in red corpuscles.

Verdohemochromogen has not been demonstrated spectroscopically in the blood stream. Its existence is assumed because of the presence of the easily split off iron of the red blood cells.

#### SPECTROPHOTOMETRIC PROPERTIES

Investigations concerning the nature of the blood pigments have depended largely upon their color, because of their relatively sharp absorption bands and the ease of spectroscopic examination. It is to be emphasized that, at the present time, the identification of the various blood pigments still depends mainly upon their spectral properties. For example, the characterization of sulphemoglobin and pseudomethemoglobin is based solely upon the position of their absorption bands and the behavior of these bands under chemical manipulation.

(a) *Theoretical*.<sup>34, 35</sup>—The change in intensity of light, as it passes through an absorbing medium, is given by the following equation:

$$- \frac{dI}{dI} = KI \quad (1)$$

Upon integrating and changing to logarithms to the base 10, we obtain,

$$- \log \frac{I_1}{I_0} = EI \quad (2)$$

where

$I_0$  = intensity of incident light.

$I_1$  = intensity of light at depth of solution 1.

$E$  and  $K$  are constants.

For monochromatic light the absorption is generally proportional to the number of molecules in the path of the light. The relationship is given by the Beer-Lambert<sup>36</sup> law which has been verified for dilute solutions of the blood pigments.<sup>37</sup>

$$- \log \frac{I_1}{I_0} = E \, c \, l \quad (3)$$

where

$E = \frac{E}{c}$  and  $c$  = concentration of absorbing material.

$E$  is known as the extinction coefficient (transmissive index) and is equal to  $-\log \frac{I_1}{I_0}$  (optical density or extinction) when the depth of the solution is unity.  $E$  is the specific extinction coefficient (specific transmissive index) and is equal to the optical density at unit depth and concentration. If  $\frac{I_1}{I_0}$  equals  $\frac{1}{10}$ , then  $E \, c \, l = 1$ , and the physical meaning of the extinction coefficients becomes

clear. The specific extinction coefficient is the reciprocal of that concentration necessary to reduce the light to  $\frac{1}{10}$  of its original intensity in a unit layer of solution.<sup>38\*</sup>

Qualitative spectrophotometry depends upon the fact that each substance exhibits a characteristic relationship between extinction and wave length, i.e., a characteristic absorption spectrum. This relationship is generally expressed by the curve obtained when the extinction coefficients are plotted as ordinates against the wave length as abscissae.<sup>39</sup> However, when the concentration is varied or when different units of concentration are used, it is found that the curves for a particular substance cannot be superimposed. The curves will vary both in form and in height, since  $E = E/C$ . This effect of concentration in changing the form of the curve is canceled if one plots the logarithm of the extinction coefficient against the wave length.<sup>34</sup> Curves for the same substance at different concentrations can then be superimposed. They run parallel to each other at an interval equal to  $\log c$ , since  $\log E = \log E - \log c$ .<sup>40</sup> By plotting the data on semilogarithm paper, one retains the simplicity of the first method and the advantages of the unchanging form of the second method ("typical absorption curves"). This type of curve becomes particularly important when the concentration of the substance is not known.

(b) *Absorption Spectra.*—The "typical absorption spectra" of some of the blood pigments and their derivatives are shown in Figs. 1 and 2. Oxyhemoglobin is easily distinguished by the characteristic bands at 540 and 575  $m\mu$ . Upon the addition of strong reducing agents (e.g., hydrosulfite) both bands disappear, to be replaced by the single strong band at 555  $m\mu$  of reduced hemoglobin. Addition of oxidizing agents (ferrieyanide) in neutral or acid solutions causes the appearance of the alpha methemoglobin band at 630 to 634  $m\mu$ . This methemoglobin band disappears upon the addition of a few grains of cyanide, hydrosulfite, sulfide, azide, fluoride, or alkali. Sulfhemoglobin has a characteristic band at 618 to 620  $m\mu$  which is unchanged by the addition of the reagents mentioned above in the case of methemoglobin. The band disappears upon the addition of ferrieyanide and is shifted from 5 to 7  $m\mu$  towards the violet when carbon monoxide is bubbled through the solution. The methemoglobin band, on the contrary, is not changed by the addition of carbon monoxide. Illuminating gas must be purified for this purpose since it may contain sulfides or reducing agents which will alter methemoglobin. Hematin in acid solution shows a strong alpha band at 660  $m\mu$ . In N/10 sodium hydroxide, the alpha band is less prominent and has its maximum at 610  $m\mu$ . When seen in serum, hematin generally shows a band at 610 to 625  $m\mu$ . The band disappears upon addition of cyanide. Cyanide and ammonium sulfide or sodium hydrosulfite disperse the band with the formation of a hemochromogen spectrum (sharp bands at approximately 560 and 530, the former band being the stronger).

(c) *Qualitative Spectroscopic Examination of the Blood.*—Freshly drawn, oxalated blood is placed in a small test tube and centrifuged. The plasma is

\*Frequent use is made of the term *absorption constant* or *absorption ratio*, which is designated by  $A$ . The definition is given by the equation

$$c = A \times E \quad (4)$$

$A$  is the reciprocal of the specific extinction coefficient (usually in units of grams solute per cubic centimeter of solution).

removed and the cells are diluted with an equal quantity of water. Hemolysis is completed by the addition of a few milligrams of saponin. The mixture is stirred cautiously since shaking produces a foam which hinders the examination. Whole blood may be hemolyzed with saponin without the addition of water if one is not interested in the plasma pigments. Illumination is furnished by a small vertical beam from a shielded 100 watt frosted bulb. Through a low dispersion, direct vision, and hand spectroscope, various thicknesses of solution are examined by tilting the test tube.\* A point will be found where only the red region of the spectrum is light enough to permit the detection of absorption bands. The 630 methemoglobin band appears to divide the red region at a point approximately  $4/10$  of the distance from the yellow end. The 620 sulfhemoglobin band is slightly nearer the alpha hemoglobin band. A practiced observer can detect this difference with the hand spectroscope even before the application of the usual confirmatory tests (disappearance of the methemoglobin band but stability of the sulfhemoglobin band on addition of a few drops of 1 per cent potassium cyanide,<sup>44</sup> dilute ammonia, or a few grains of sodium hydrosulfite). The band of sulfhemoglobin can be abolished by the addition of ferricyanide, but cyanide must be added to prevent the appearance of the methemoglobin absorption in that region. If hydrosulfite is now added, the sulfhemoglobin band at 620 returns.

The sensitivity of this test does not depend upon the absolute concentration of the abnormal pigment but on the relative intensities of the abnormal band and the diffuse absorption of oxyhemoglobin in that portion of the spectrum. Methemoglobin can be detected when it constitutes as little as 3 to 4 per cent of the total pigment (approximately 0.5 gm. per cent).<sup>45</sup> Since the sulfhemoglobin band is about three times as strong as that of methemoglobin, 1 per cent sulfhemoglobin in normal blood (0.1 gm. per cent) can be seen.<sup>43a</sup> The simultaneous occurrence of sulfhemoglobin and methemoglobin is indicated when the band in the red becomes lighter but does not disappear upon the addition of cyanide, and seems to be shifted toward the blue end of the spectrum.

Examination of the plasma can be carried out in similar fashion. Slight hemolysis will cause the appearance of the two banded oxyhemoglobin spectrum. Methemoglobin is detected as described above. Hematin produces a band at approximately 610 to 630  $m\mu$ . Upon the addition of cyanide, this band disappears. Upon the further addition of ammonium sulfide, a hemochromogen spectrum, with bands at 530 and 560, appears. This serves to distinguish clearly between methemoglobin and hematin, since methemoglobin reverts to reduced hemoglobin under these conditions. Pseudomethemoglobin shows a band at 622 which does not change on addition of Stokes' reagent or 10 per cent ammonium sulfide, but disappears upon the addition of sodium hydrosulfite.

\*Greater elegance in this examination may be achieved through the use of easily constructed apparatus.<sup>43</sup> The use of a glass wedge simplifies the examination of various depths of solution.

## QUANTITATIVE DETERMINATION OF THE BLOOD PIGMENTS

Methods for the quantitative determination of the blood pigments may be divided into three general types: gasometric, combined gasometric and colorimetric, and spectrophotometric.

(a) *Gasometric*.—Gasometric methods have been applied only to the determination of hemoglobin and methemoglobin. They are based upon the determination of the oxygen or carbon monoxide combining power of blood before and after the conversion of methemoglobin into hemoglobin through the action of reducing agents. The carbon monoxide capacity method was first suggested by Nieloux and Fontes<sup>46</sup> and elaborated by Van Slyke and his co-workers.<sup>47</sup> This method is given in detail by Peters and Van Slyke.<sup>48</sup> The oxygen capacity method was improved by Conant and Fieser.<sup>49</sup> The oxygen capacity is determined by the Van Slyke and Neill<sup>50</sup> method before and after reduction with sodium anthrahydroquinone B sulfonate<sup>49</sup> or titanous tartrate.<sup>51</sup>

The gasometric methods require experience and are tedious. The oxygen capacity method is specific for methemoglobin, but there is always the danger of the reoxidation of the laked hemoglobin to methemoglobin during the aeration with oxygen. This tends to make the final result too low, but the error is minimized by the use of titanous tartrate as the reducing agent. As Conant<sup>51</sup> has pointed out, the carbon monoxide methods are disturbed by the presence of other pigments. After reduction, both hematin and sulfhemoglobin may combine with carbon monoxide and thus appear in the result as methemoglobin. In both methods methemoglobin is calculated from the difference between two determinations, thus magnifying the errors. There are no gasometric methods for sulfhemoglobin or other pigments.

(b) *Combined Gasometric and Colorimetric Determinations*.—Active hemoglobin is determined by the carbon monoxide or oxygen combining power. Total pigment is determined by a variety of methods: colorimetric cyanmethemoglobin,<sup>52, 53</sup> acid hematin,<sup>54</sup> methemoglobin,<sup>55</sup> or total iron.<sup>56</sup> The difference between the active hemoglobin and the total pigment represents the methemoglobin or sulfhemoglobin.

These methods are not specific. If the oxygen capacity is used for the determination of the active hemoglobin and the total iron for total hemoglobin, the result will represent all other iron-containing pigments: methemoglobin, sulfhemoglobin, hematin, and some nonhemoglobin iron. In the case of the carbon monoxide methods, a variable amount of sulfhemoglobin may combine with the gas and be calculated as active hemoglobin. The difficulties of the colorimetric methods have been discussed by Schwentker.<sup>57</sup> They require fresh standards which are difficult to prepare and must be constantly rechecked. Turbidity,<sup>58</sup> hematin, or other colored substances interfere with the cyanmethemoglobin and methemoglobin methods. The iron analyses are more accurate but are more difficult to conduct and generally require preliminary ashing.

When both methemoglobin and sulfhemoglobin are present, the only combined method would require the determination of the difference in oxygen capacity before and after reduction with titanous tartrate as a measure of

methemoglobin followed by the determination of total pigment by the acid hematin or iron analytic methods to obtain the sulphemoglobin concentration. Nonhemoglobin iron naturally interferes.

(c) *Spectrophotometric Methods*.<sup>34, 35, 59-61\*</sup>—Quantitative spectrophotometry depends upon the equations previously derived, and the fact that the total extinction coefficient for a solution is an additive function of the separate extinction coefficients of its constituents in the absence of interaction.

$$E_t = E_1 + E_2 \quad (5)$$

Utilizing these principles, the following spectrophotometric methods have been evolved:

(1) The extinction coefficient of a solute may be calculated by subtraction of the extinction of the remainder of the materials in solution from the total observed extinction. This operation is performed automatically if one places the solution containing the compound in question in one cup of the spectrophotometer and the solution without the compound in the other cup. The concentration of the solute may be readily determined from the observed extinction if the specific extinction coefficient is known.

$$\text{conc. (x)} = E \text{ (observed)} / E_x \quad (6)$$

(2) Method of Vierordt.<sup>59, 62</sup> The concentrations of two colored substances in an optically clear solution may be calculated from the total extinction coefficients of the solution at two wave lengths through the use of simultaneous equations.

Let

$E_{am}$  and  $E_{bm}$  be the extinction coefficients of the solution at wave lengths  $a$  and  $b$ .

$E_{ax}$ ,  $E_{ay}$ ,  $E_{bx}$ , and  $E_{by}$  be the known specific extinction coefficients of components  $x$  and  $y$  at wave lengths  $a$  and  $b$ .

$X$  and  $Y$  be the concentrations of components  $x$  and  $y$  in the solution.

From 5 and 6 we obtain

$$E_{am} = E_{ax} X + E_{ay} Y \quad (7)$$

$$E_{bm} = E_{bx} X + E_{by} Y \quad (8)$$

Substituting and rearranging

$$X = \frac{E_{by} E_{am} - E_{bm} E_{ay}}{E_{by} E_{ax} - E_{ay} E_{bx}} \quad (9)$$

$$Y = \frac{E_{ax} E_{bm} - E_{am} E_{bx}}{E_{by} E_{ax} - E_{ay} E_{bx}} \quad (10)$$

These principles may be extended to solutions containing three or more solutes. Application and criticism of this method will be discussed with those of the following procedure.

\*The chief drawback to spectrophotometric methods is the expense of the apparatus. Although we realize that these instruments are by no means in widespread clinical use, the principles involved have been explained fully since they form the basis of all colorimetric methods.

Recently photoelectric photometers have become increasingly popular because of their convenience, simplicity, and accuracy. All methods used in these instruments are based upon spectrophotometric principles, and the spectrophotometer is essential in the development of new applications. Conversely, all spectrophotometric methods are easily adapted to the photoelectric photometers if one has the choice of suitable, narrow banded filters. It is only necessary to substitute the apparent extinction coefficients of the substances with the proper filter for the true spectrophotometric extinction coefficients.

(3) Method of Hufner.<sup>63</sup> Hufner's method is very similar to that of Vierordt. The same simultaneous equations are used, but the final formula gives the *percentage composition* of the mixture in terms of the ratio (R) of the extinction coefficients of the mixture at two wave lengths.

Let  $R = E_{nm} / E_{lm}$

and per cent X =  $100 X / X + Y$  (percentage concentration of X in the total colored solutes).\*

From 7 and 8 one obtains the following equation by division and rearrangement:

$$\text{Per cent X} = \frac{100 (E_{nx} - R E_{lx})}{R (E_{bx} - E_{ly}) + E_{ny} - E_{nx}} \quad (11)$$

Several methods have been proposed for the determination of methemoglobin in the presence of oxyhemoglobin through the use of these formulas. Most of these depend upon the Hufner ratio of the extinction coefficients at 540/560,<sup>64</sup> which have been determined for both oxyhemoglobin and alkaline methemoglobin by Hari<sup>39, 65</sup> and Heilmeyer.<sup>34</sup> However, these ratios are so close together that a large change in the composition of a mixture is reflected by only a small change in the ratio, e.g. 0.43 for the complete conversion of oxyhemoglobin to methemoglobin. Both Heilmeyer and Drabkin and Austin<sup>66</sup> point out that the ratios at 576-575/560  $m\mu$  change approximately twice as much, for a given change in composition, as those at 540/560  $m\mu$ , and Heilmeyer<sup>34</sup> shows that the ratios at 576-575/590 have a far greater dispersion. The choice of wave lengths at 540  $m\mu$  and 560  $m\mu$  is doubly disadvantageous since Austin and Drabkin have demonstrated that this ratio is markedly influenced by pII variations.<sup>67</sup> Their data show that both the ratios at 576/560 and at 576/590 are much less disturbed by changes in the pII of the solution. The disadvantage of the 590  $m\mu$  measurement is that the light transmissions of both oxyhemoglobin and methemoglobin are changing very rapidly with wave length.

Because these methods depend to a variable extent upon the pH of the solution, Michel<sup>68</sup> has advised that the methemoglobin be converted to cyanmethemoglobin. The oxyhemoglobin is not changed by this procedure and the absorption spectra of both oxyhemoglobin<sup>69</sup> and cyanmethemoglobin are nearly independent of pII variations. The ratios of the extinction coefficients for these substances at 575  $m\mu$  and 560  $m\mu$  vary even more, for the same change in composition, than the corresponding ratios for oxyhemoglobin and methemoglobin. This method will be described in detail later.

For the determination of the total pigment in the mixture, the equations of Vierordt may be used. Drabkin and Austin<sup>66</sup> believe that greater accuracy is obtained if the total pigment is measured separately by the absorption at any wave length after conversion to cyanmethemoglobin by the addition of ferricyanide and cyanide.

The above methods are rapid but their accuracy is disturbed by any turbidity of the solution or the presence of a third pigment. To clarify the

\* Hereafter percentage concentration refers to this concept. Concentration will refer to grams of the component in 100 c.c. solution.

solutions for the determination of the percentage methemoglobin, we fractionally precipitate with ammonium sulfate and use the filtrate. This procedure does not affect the methemoglobin-hemoglobin ratio of the mixture or cause any detectable loss in pigments.

Methods for the determination of sulfhemoglobin in oxyhemoglobin solutions have been worked out along similar lines. Prior to 1935, spectrophotometric methods could not be used since the absorption curve of sulfhemoglobin had not been determined. This curve has now been determined indirectly.<sup>9</sup> Our method for the determination of sulfhemoglobin depends upon the ratio of the extinction coefficients of the mixture of oxyhemoglobin

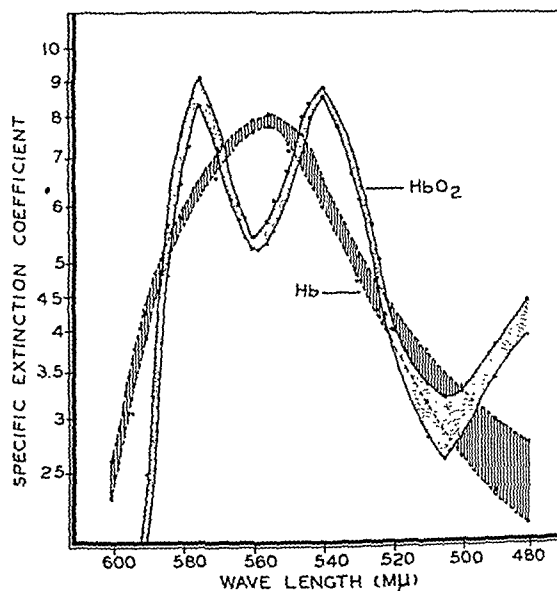


Fig. 1.—The "typical absorption spectra" of oxyhemoglobin and hemoglobin. Oxyhemoglobin curve plotted from the data of Hari,<sup>30</sup> Charnass,<sup>41</sup> Kennedy,<sup>38</sup> and Drabkin and Austin.<sup>4, 5</sup> Hemoglobin curve plotted from the data of Warburg, Negelein, and Christian,<sup>39</sup> Hari,<sup>42</sup> and Drabkin and Austin.<sup>5</sup> The shaded areas in each curve represent the maximum deviations found. Ordinate: Specific extinction coefficient for a 1 gm. per 100 c.c. solution. Abscissa: Wave length in  $m\mu$ .

and sulfhemoglobin at 540  $m\mu$  / 620  $m\mu$ . The values of these extinction coefficients were taken from the data of Drabkin and Austin.<sup>9</sup> The absorption spectra of both compounds are only slightly influenced by pH changes but, as in the other methods, the solutions must be optically clear. Although the method requires the reading of the extinction at 620  $m\mu$  in concentrated solution and then the dilution of the solution in order to obtain the extinction at 540  $m\mu$ , it has the advantage of giving large changes in the ratio for very small changes in the percentage concentration of sulfhemoglobin.

(4) Method of Haurowitz.<sup>28</sup>—The concentration of each of two colored substances in solution can be calculated if the total concentration of pigment is known and the total extinction of the mixture is determined at any wave length.

Let  $C_t$  be the total concentration of pigment.

$E_m$ , X, Y, etc., be as in equations 7 and 8.

From 5 and 6,

$$E_m = E_x X + E_y (C_t - X) \quad (12)$$

and

$$X = \frac{E_m - E_y C_t}{E_x - E_y} \quad (13)$$

Haurowitz<sup>25</sup> noted that the absorption of methemoglobin at 623  $m\mu$  was independent of the pH of the medium. He, therefore, proposed that the extinction coefficients at 623  $m\mu$  of a mixture of methemoglobin and oxyhemoglobin be determined before and after the conversion of all pigment to methemoglobin by means of ferrieyanide. The total pigment is determined by the division of the extinction coefficient after ferrieyanide addition by the specific extinction coefficient of pure methemoglobin. The concentration of methemoglobin in the original mixture is then obtained from formula 13, using the extinction coefficient of the mixture before ferrieyanide as the  $E_m$  value. The method, however, is inaccurate because of the effect of turbidity and because the isobestic point may not be located exactly at 623  $m\mu$ .

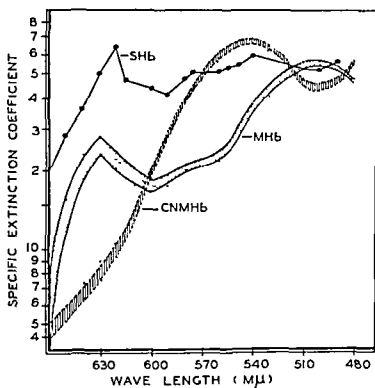


Fig. 2.—The "typical absorption spectra" of acid methemoglobin, cyanmethemoglobin, and sulfhemoglobin. Acid methemoglobin curve constructed from the data of Hari,<sup>68</sup> Haurowitz,<sup>25</sup> and Austin and Drabkin.<sup>67</sup> Cyanmethemoglobin curve from data of Haurowitz,<sup>25</sup> and Drabkin and Austin.<sup>9</sup> Sulfhemoglobin curve after Drabkin and Austin.<sup>9</sup> Ordinate and abscissa as in Fig. 1.

Austin and Drabkin<sup>67</sup> have advocated the use of a similar method for the determination of methemoglobin in oxyhemoglobin solutions. Their method requires the following measurements: The extinction coefficients of the solution at 630  $m\mu$ , 575  $m\mu$ , 560  $m\mu$ , and 540  $m\mu$ ; the pH; and the concentration of total pigment by conversion to cyanmethemoglobin as previously described. The solutions must be optically clear and the absorption must be due only to methemoglobin and oxyhemoglobin. Turbidity or a third pigment will falsify the result.

(5) Authors' method. If the composition of one colored component in a mixture can be altered without changing any of the other components, the concentration of that substance may be determined by measuring the extinction coefficient of the mixture before and after the alteration.



Let

$E_{ax}$  be the specific extinction coefficient of component X at wave length a.  
 $E_{ax}'$  be the specific extinction coefficient of substance X' which is formed from X by the addition of Z.

$E_m$  be the observed extinction coefficient of the original mixture.

$E_z$  be the observed extinction coefficient of the mixture after the addition of Z.

Then

$$\text{Conc. X} = \frac{E_m - E_z}{E_{ax} - E_{ax}'} \quad (14)$$

This method has been applied to the photoelectric photometer determination of methemoglobin in oxyhemoglobin solutions by Evelyn and Malloy<sup>17</sup> and independently by us to the spectrophotometric determination. Both are based upon the fact that the addition of cyanide quantitatively converts methemoglobin to cyanmethemoglobin without affecting the absorption spectra of either sulfhemoglobin or oxyhemoglobin. Because the spectrum of methemoglobin varies with the pH and ionic strength, it is necessary to buffer the solution to a known pH and to a reasonably constant salt concentration. The buffer is also necessary to prevent the cyanide from changing the clarity of the solution. With these restrictions, the methods are simple; they allow the use of uncleared solution and may be used in the presence of other pigments which are unaffected by cyanide.

#### METHODS

##### A. The Determination of Methemoglobin in Oxyhemoglobin Solutions by the Use of the Hufner Ratio for Cyanmethemoglobin and Oxyhemoglobin

One volume of solution (whole oxalated blood) is hemolyzed by the addition of a few milligrams of saponin. Two volumes of half saturated ammonium sulfate are added. The mixture is cooled and filtered. The filtrate is diluted with distilled water until the total pigment concentration is approximately 0.1 gm. per cent. The final dilution of whole oxalated blood should be 1:100. Approximately 2 to 3 mg. of dry potassium cyanide are added, and the extinction coefficients are read at wave lengths 575 and 560  $m\mu$ . The ratio (R) of the extinction coefficient at 575  $m\mu$  to that at 560  $m\mu$  is calculated. The fraction of total hemoglobin in the form of methemoglobin is given by the following equation.

$$\text{MHB/Total HB} = \frac{9.06 - 5.39 R}{0.53 + 4.66}$$

This value may be obtained directly from the calibration curve in Fig. 3.

It has been stated previously that the R value of a solution is independent of concentration. If one is merely interested in the fraction of total hemoglobin in the form of methemoglobin, the dilutions may be carried out very roughly. The method is, therefore, useful for the rapid determination of the percentage composition of a series of hemoglobin solutions. To determine the total hemoglobin, the dilutions must be made accurately. The total concentration may be calculated from the following equation:

$$\text{Grams per cent total pigment} = \frac{4.66 E_{560} + 0.53 E_{575}}{29.91} \times \frac{1}{\text{Final dilution}}$$

The results may be checked by converting all the pigment to cyanmethemoglobin and then determining the extinction coefficient at 540  $m\mu$ . After the readings at 575 and 560 have been taken, approximately 3 mg. of dry potassium ferrieyanide are added to the material in the spectrophotometer cup. A few minutes later, an additional 2 to 3 mg. of potassium cyanide are added. The extinction coefficient is read at 540  $m\mu$ .

$$\text{Grams per cent total pigment} = \frac{E_{510}}{6.9} \times \frac{1}{\text{Final dilution}}$$

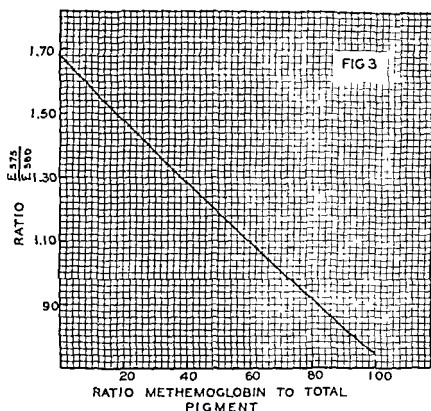


Fig. 3.—The values of the ratio of the extinction coefficients at 575  $m\mu$  to that at 560  $m\mu$  for varying mixtures of cyanmethemoglobin and oxyhemoglobin

#### B. The Determination of Methemoglobin and Oxyhemoglobin by Means of the Change in Extinction on Conversion to Cyanmethemoglobin

One cubic centimeter of whole oxalated blood is hemolyzed by the addition of a few milligrams of saponin. It is then diluted to approximately 23 c.c. with distilled water. One and one-fourth cubic centimeters of 1.0 molar phosphate buffer\* are added, and the solution diluted to the final volume of 25 c.c. Without filtering, the extinction coefficient is determined at 634  $m\mu$ . Two to 4 mg. dry potassium cyanide are added to the solution in the spectrophotometer cup and, one minute later, the extinction coefficient at 634  $m\mu$  is again determined. It is to be noted that it is unnecessary to read the solution through both beams of the photometer, since one is only interested in the difference between the two extinctions and not their absolute values. The cells and plungers of the spectrophotometer must be carefully washed after the use of cyanide, since the next sample may be partly changed by the

\*1 M 7.4 Phosphate buffer: 114.8 gm. anhydrous  $\text{Na}_2\text{HPO}_4$  and 26.15 gm. anhydrous  $\text{KH}_2\text{PO}_4$  per liter.

1 M 6.2 Phosphate buffer: 26.42 gm.  $\text{Na}_2\text{HPO}_4$  and 110.9 gm.  $\text{KH}_2\text{PO}_4$  per liter.

The factors for two phosphate buffers have been given because it is sometimes necessary, as in tissue respiration experiments, to buffer the solution to pH 7.4. At this pH, approximately  $\frac{1}{2}$  of the methemoglobin is in the alkaline form and the factor is, therefore, smaller than that at pH 6.2.

cyanide before the first extinction is read. The concentration of methemoglobin is given by the following formula:

$$\text{Grams per cent methemoglobin} = \frac{E_{634} - E_{634} \text{ with cyanide}}{F}$$

where  $F$  is 0.0728 when pH of solution is 7.4, or 0.0860 at pH 6.2.

To determine the total hemoglobin and methemoglobin concentration, approximately 5 mg. of dry potassium ferricyanide are added to another portion of the diluted blood. After mixing, the solution is allowed to stand five minutes. The extinction coefficients at 634  $m\mu$  are then obtained before and after the addition of cyanide. The total pigment is calculated by the same formula as above.

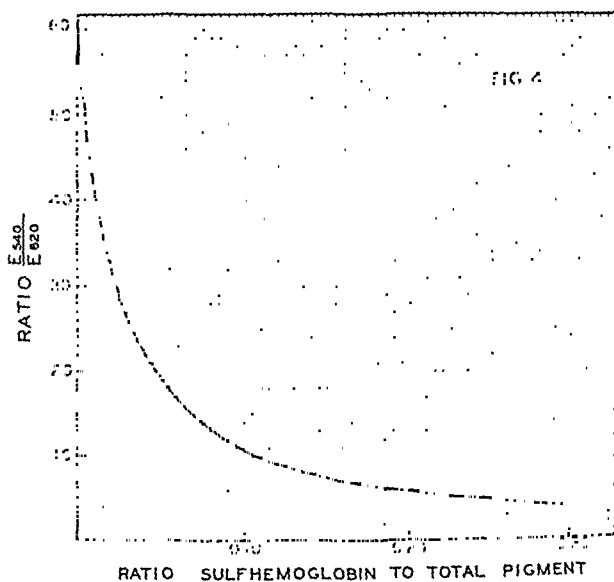


Fig. 4.—The values of the ratio of the extinction coefficients at 540  $m\mu$  to that at 620  $m\mu$  for varying mixtures of sulfhemoglobin and oxyhemoglobin.

### C. The Determination of Sulfhemoglobin

Methemoglobin is first determined by a modification of the preceding method. The blood is, therefore, clarified by the use of 2 M pH 7.4 phosphate buffer\* instead of ammonium sulfate. The greater ionic strength of this solution necessitates a change in the factor used in the calculation of methemoglobin concentration. After the extinctions at 634  $m\mu$  have been determined, the ratio of the extinction coefficients at 540  $m\mu$  to that at 620  $m\mu$  is obtained in order to calculate the sulfhemoglobin concentration according to the usual formulas. The presence of the cyanide does not affect the extinctions of either the oxyhemoglobin or sulfhemoglobin, but corrections must be introduced to account for the presence of the cyanmethemoglobin. If methemoglobin is not present in the original solution, the first part of the procedure and the corrections may be omitted.

One volume of solution (whole oxalated blood) is hemolyzed by the addition of a few milligrams of saponin. Two volumes of 2 molar pH 7.4

\*2 M pH 7.4 Phosphate buffer: 52.30 gm.  $\text{KH}_2\text{PO}_4$  and 281.68 gm.  $\text{K}_2\text{HPO}_4$  per liter.

phosphate buffer are added, and the mixture is filtered. Two cubic centimeters of the filtrate are diluted to 10 c.c. with distilled water. The extinction is determined at 634  $m\mu$  before and after the addition of 2 to 4 mg. of potassium cyanide. The extinction coefficient at 620  $m\mu$  is then determined. One cubic centimeter of the material in the spectrophotometer cup is now diluted to 10 c.c. (making a 1:150 dilution of the original blood), and the extinction coefficient is determined at 540.

Calculations:

$$\text{Grams per cent methemoglobin} = \frac{E_{634} - E_{634} \text{ with KCN}}{0.126}$$

$$\text{Corrected 620 extinction} = 15 E_{620} - 0.09 \times \text{gm. per cent MHB}$$

$$\text{Corrected 540 extinction} = 150 E_{540} - 6.9 \times \text{gm. per cent MHB}$$

From the corrected readings, the ratio (R) of the extinction at 540 to that at 620 is calculated and used in the following formula to determine the ratio of pigment (exclusive of methemoglobin) in the form of sulfhemoglobin.

$$\frac{\text{Sulfhemoglobin}}{\text{Total hemoglobin}} = \frac{8.76 - 0.144 R}{6.26 R + 2.86}$$

The percentage composition (exclusive of methemoglobin) may be read directly from the calibration curve in Fig. 4.

The total pigment concentration in grams per 100 c.c. of the original solution is calculated from the corrected extinction coefficients by means of the following equation:

$$\text{Grams per cent total pigment} = \text{Grams per cent methemoglobin} + \frac{6.26 E_{540} + 2.86 E_{620}}{55.2}$$

#### EXPERIMENTAL

The total hemoglobin content of freshly drawn oxalated human blood was determined after reduction with sodium hydrosulfite by the carbon monoxide capacity method of Van Slyke and Hiller.<sup>47</sup> Solutions of these bloods were then prepared in accordance with the methods of determination previously described. The extinctions at the wave lengths used in the various methods were then obtained on the Bausch and Lomb Universal Spectrophotometer.\* Another sample of the original blood was then checked for the active hemoglobin content by the carbon monoxide method without preliminary reduction with hydrosulfite. In this manner, the absence of methemoglobin in the original sample was assured. Spectroscopic analyses ruled out the presence of other abnormal pigments. One experiment (Blood No. 9, Table I) was performed on purified hemoglobin prepared by shaking washed red blood cells with toluene, centrifuging off the cell debris, and dialyzing the solution against distilled water for several days. The results are tabulated in Table I, and the values found by other investigators on differently prepared solutions are given for comparison.

\*The technique of using this apparatus is fully described by Hellmeyer<sup>48</sup> and Kennedy.<sup>49</sup> For the work herein reported, the apparatus was placed in an air-conditioned darkroom at 20° C. The spectroscopic was calibrated by means of the sodium light and the arc spectra of lithium, potassium, and magnesium. The solutions were observed in both beams of light, and the arithmetical means of the observed extinctions were calculated. The depth of solution was such that the observed extinctions were between 0.50 and 1.50, which Kennedy found to be the optimum range. The spectroscopic objective slit was reduced to a minimum, and the portion of spectrum observed was always less than 4  $m\mu$ .

TABLE I  
EXTINCTION COEFFICIENTS OF OXYHEMOGLOBIN AND CYANHEMOGLOBIN

BLOOD SAMPLES										VALUES IN LITERATURE						
	1	2	3	4	5	6	7	8	9	AVER- AGE	HART <sup>32</sup>	NEW- COMER <sup>31</sup>	DRAB- KIN AND AUSTIN <sup>9</sup>	KEN- NEDY <sup>33</sup>	CHAR- NASS <sup>41</sup>	MAR- TINI <sup>72</sup>
CO combining power	22.36	19.36	21.37	19.57	24.13	22.35	17.74	18.95	24.85	--	--	--	--	--	--	--
Hemoglobin (gm. %)	16.66	14.43	15.93	14.60	17.99	16.66	13.23	14.12	18.52	--	--	--	--	--	--	--
HbO <sub>2</sub> E <sub>410</sub> 1:100 dil.	1.440	1.255	1.374	1.302	1.595	1.464	--	--	--	--	--	--	--	--	--	--
HbO <sub>2</sub> E <sub>540</sub>	8.65	8.70	8.63	8.92	8.87	8.79	--	--	--	8.76	8.50	8.79	8.80	8.59	8.55	9.14
HbO <sub>2</sub> E <sub>520</sub> 1:100 <sup>9</sup> dil.	0.896	0.766	0.842	0.801	0.978	0.903	--	--	--	--	--	--	--	--	--	--
HbO <sub>2</sub> E <sub>520</sub>	5.38	5.31	5.28	5.49	5.44	5.42	--	--	--	5.39	5.28	5.34	5.28	5.26	5.18	5.55
HbO <sub>2</sub> E <sub>535</sub> 1:100 <sup>9</sup> dil.	1.498	1.296	1.412	1.352	1.653	1.508	--	--	--	--	--	--	--	--	--	--
HbO <sub>2</sub> E <sub>535</sub>	9.00	8.98	8.87	9.26	9.19	9.06	--	--	--	9.06	8.35	9.36	9.06	8.70	8.64	9.43
HbO <sub>2</sub> E <sub>535</sub> 1:10 <sup>9</sup> dil.	0.243	0.209	0.228	0.212	0.250	0.245	--	--	--	--	--	--	--	--	--	--
HbO <sub>2</sub> E <sub>535</sub>	0.146	0.145	0.143	0.145	0.139	0.147	--	--	--	0.144	0.116	0.467	0.33	Hauro- witz <sup>33</sup>	--	--
CNMMHb E <sub>425</sub>	--	0.621	0.692	0.544	0.804	0.750	0.581	--	--	--	--	--	--	--	--	--
1:100 dil.	--	4.30	4.34	4.41	4.47	4.50	4.39	--	--	4.40	--	--	4.46	4.17	--	--
CNMMHb E <sub>425</sub>	--	0.833	0.928	0.878	1.088	0.992	0.784	--	--	--	--	--	--	--	--	--
1:100 dil.	--	5.77	5.82	6.01	6.05	5.95	5.92	--	--	5.92	--	--	5.97	5.63	--	--
CNMMHb E <sub>520</sub>	1.801	--	1.897	1.831	1.821	--	1.845	1.812	1.809	1.82	--	--	--	--	--	--
F pH 7.4 0.05 M buffer	2.138	--	2.165	2.150	2.170	--	2.110	2.152	2.160	2.15	--	--	--	--	--	--
F pH 6.2 0.05 M buffer	--	--	--	--	--	--	--	--	--	1.89*	--	--	--	--	--	--
F pH 7.4 0.27 M buffer	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Note: F = Change in E at 631 mμ on converting methemoglobin to cyanmethemoglobin. E given for 1 gm. in 100 c.c. of solution.  
\*Value obtained by determining F of 5 solutions at pH 7.4 at 0.95 M and 0.27 M buffer.

Tables II and III show the results obtained with known solutions of methemoglobin and oxyhemoglobin. They were prepared by mixing definite amounts of two solutions whose oxyhemoglobin and methemoglobin contents were determined by the gasometric carbon monoxide method of Van Slyke and Miller.<sup>47</sup> Table III gives the results obtained by means of the difference in extinction before and after conversion of the methemoglobin to cyanmethemoglobin, while Table II gives the results obtained by means of "Hufner ratio" for oxyhemoglobin and cyanmethemoglobin. The former method is considerably more accurate than the latter and has the advantage that the accuracy can be improved by reading the extinctions in more concentrated solutions when the amount of methemoglobin is small. Both methods require few reagents, and many analyses can be carried out within an hour.

TABLE II

DETERMINATION OF METHEMOGLOBIN BY MEANS OF THE RATIO OF EXTINCTION COEFFICIENTS AT 575 AND 560  $m\mu$  AFTER ADDITION OF CYANIDE

SOLUTION	GASOMETRIC CARBON MONOXIDE METHOD			SPECTROPHOTOMETRIC METHOD	
	TOTAL HEMO-GLOBIN (GM. %)	METHEMO-GLOBIN (GM. %)	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN	RATIO EXTINCTION AT 575 TO 560 $m\mu$	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN
1	13.2	0.3	2.3	1.66	2.0
2	18.5	5.9	31.9	1.35	33.0
3	13.9	1.0	7.2	1.59	8.6
4	14.6	1.7	11.6	1.56	12.0
5	15.2	2.4	15.8	1.53	14.8
6	15.9	3.1	19.5	1.48	19.8
7	16.5	3.8	23.0	1.44	23.8
8	17.2	4.5	26.2	1.41	26.9
9	17.9	5.2	29.0	1.37	31.0

TABLE III

DETERMINATION OF METHEMOGLOBIN BY CHANGE IN EXTINCTION UPON CONVERSION TO CYANMETHEMOGLOBIN

SOLUTION	GASOMETRIC CARBON MONOXIDE METHOD			SPECTROPHOTOMETRIC METHOD		
	TOTAL HEMO-GLOBIN (GM. %)	METHEMO-GLOBIN (GM. %)	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN	TOTAL HEMO-GLOBIN (GM. %)	METHEMO-GLOBIN (GM. %)	PER CENT TOTAL PIGMENT PRESENT AS METHEMOGLOBIN
1	13.2	0	0	13.2	0	0
2	18.5	5.71	30.8	18.6	5.55	29.8
3	13.9	0.71	5.1	13.7	0.71	5.2
4	14.6	1.43	9.7	14.5	1.41	9.7
5	15.2	2.14	14.1	15.3	2.16	14.1
6	15.9	2.86	18.0	15.8	2.80	17.7
7	16.5	3.57	21.6	16.5	3.63	22.0
8	17.2	4.28	24.9	17.1	4.29	25.0
9	17.9	5.00	27.9	17.8	4.97	27.9

The procedure for sulfhemoglobin has not been checked because of the lack of methods. It would be pointless to check our method by means of other spectrophotometric methods which depend upon the same constants of Drabkin and Austin. Gasometric and other analytic methods are valueless for this purpose.<sup>9</sup>

## SUMMARY

The interrelationships, properties, and reactions of the clinically important blood pigments have been reviewed. The methods for the determination of sulfhemoglobin and methemoglobin have been presented and critically discussed. Simple and accurate spectrophotometric methods for the determination of sulfhemoglobin, methemoglobin, and oxyhemoglobin in mixtures have been described.

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# RELATION OF THE DIETARY CALCIUM-PHOSPHORUS RATIO TO IRON ASSIMILATION\*

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CONSIDERABLE attention has been given to the possible relationship between infantile rickets and anemia. Findlay<sup>1</sup> was the first to show that anemia in rickets was due to complicating factors such as poor nutrition and not due directly to injury of the bone marrow. He showed that children fed good diets low in vitamin D developed rickets without showing anemia. Later McDonough and Borgen<sup>2</sup> found that the administration of vitamin D to infants suffering from anemia and rickets did not raise the hemoglobin level. The administration of iron salts to the rachitic infants without supplementing the diet with cod-liver oil raised the hemoglobin content of the blood to a satisfactory level.

Working with animals, Happ<sup>3</sup> found that a severe rachitogenic diet had no effect upon the hemoglobin level of either young or mature rats. On the other hand, diets low in calcium and vitamin D, but high in phosphorus, produced both rickets and anemia, a syndrome apparently comparable to that of human infantile rickets. The observation suggested that the hemoglobin regeneration was retarded by either high level of phosphorus or low level of calcium.

There is ample evidence<sup>4, 5</sup> that a high intake of iron will bind the phosphorus and make it unavailable to the animal. From a strictly chemical point of view there is no reason to suspect that high levels of phosphorus should prevent iron assimilation, yet Day and Stein<sup>6</sup> have produced a hypochromic anemia and polycythemia in rats on low calcium-high phosphorus rations.

That excess calcium will inhibit the assimilation of iron has been recognized by Kletzein<sup>7, 8</sup> who found that in addition to calcium carbonate the carbonates of sodium, potassium, beryllium, strontium, and barium exhibit this effect.

We have been studying the effect of the calcium and phosphorus ratio on iron assimilation and utilization for some time, and in this paper we wish to report some of this work, together with an attempt to correlate our results with those obtained by other workers.

## EXPERIMENTAL

Rats were made anemic by the method of Elvehjem and Kemmerer<sup>9</sup>. When the animals were distinctly anemic, they were transferred to a dry ration

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containing varying amounts of calcium, phosphorus, iron, and copper. The basal ration had the following composition.

Corn, yellow	50
Corn, white	20
Casein	18
Lard	5
NaCl	1
Liver extract (Lilly)	2
CaCO <sub>3</sub>	0.4
NaH <sub>2</sub> PO <sub>4</sub>	0.1
Thiamine	100 micrograms

Percomorph oil was administered orally at the rate of 2 drops per week.

Care was taken in the selection and grinding of corn to reduce the iron contamination to a minimum. Several attempts were made to purify the casein, but the method effective for the removal of the iron reduced the nutritive value of the casein. In the work reported, therefore, crude casein was used. The liver extract added some iron, but its use as a source of the vitamin B complex was essential.

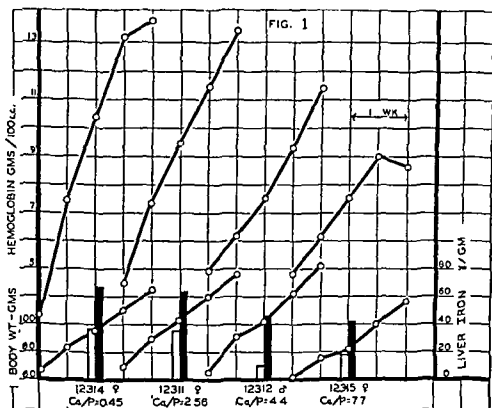


Fig. 1.—Upper curves represent hemoglobin response; lower curves, growth. Bars represent the level of total and available iron in the livers. Levels of minerals in the ration were: Fe = 139  $\mu$ g per gm. and Cu = 9.5  $\mu$ g per gm.

The basal ration never contained more than 39 micrograms of available iron per gram. Additional iron to make the desired intake was added as ferrie chloride or ferrie pyrophosphate. The copper content was varied by the addition of copper sulfate. The calcium and phosphorus were added as calcium carbonate, and sodium diphosphate, respectively.

The animals were housed in draw cages with mesh bottoms of two squares to the inch. Feed and water were given in earthen dishes with aluminum guard covers to reduce waste. The animals were weighed, and the hemoglobin content of the blood determined biweekly. They were continued on experiment for two weeks, at which time they were killed by decapitation and their livers

removed for analysis of total iron by the method of Lintzel<sup>10</sup>, and available iron by the method of Borgen and Elvehjem<sup>11</sup>.

At the culmination of the experiment the pH was determined on three segments of the gastrointestinal tract of each animal. These segments were (a) the duodenum and one-half the jejunum, (b) one-half the jejunum and the ileum, (c) the colon and the cecum. The segments were removed, their contents washed into a clean tube, diluted to 15 c.c., and the pH read on a Coleman pH electrometer.

The effect of the calcium-phosphorus ratio on iron utilization when the iron and copper levels remained constant is shown in Fig. 1. The only variable in these experiments was the calcium and phosphorus levels. Hemoglobin regeneration and iron storage in the liver were greatest on the lowest calcium-phosphorus ratio (0.45). As the calcium-phosphorus ratio was increased, there was a corresponding decrease in both hemoglobin regeneration and iron storage in the liver. Since these data are representative of the results in general, more extensive data are omitted. It serves to show that within the range of this study, phosphates have a stimulating and not an inhibitory effect upon iron assimilation.

The rapid hemoglobin response obtained on the dry ration made it seem desirable to compare the hemoglobin response on this ration with that on mineralized milk. Results shown in Fig. 2 indicate that the hemoglobin response on mineralized milk was greater than that on the dry ration which had higher calcium-phosphorus ratios, but less than that on the ration with the calcium-phosphorus ratio of 0.45. Growth on the mineralized milk used was definitely inferior to that on the dry rations.

Since blood volume correlates roughly with the body weight in a growing animal, it seemed possible that the rats on mineralized milk might be producing less hemoglobin than a litter mate which grew more rapidly, and yet show a higher hemoglobin level. Since such a condition was not easily shown by hemoglobin changes alone the following procedure was employed.

The two places for storage of iron, which have been shown to be greatly affected during the production and cure of anemia, are the liver and the hemoglobin of the blood. From the standpoint of quantity, the hemoglobin accounts for the greater portion of the iron, but the liver iron serves to indicate whether iron has not been assimilated or merely not converted to hemoglobin.

The amount of available iron consumed by the animal was determined by analyzing the ration and multiplying by the amount of ration consumed. The amount utilized was determined as follows:

To determine the amount of iron used in the formation of hemoglobin, the levels of hemoglobin, as determined by analysis, were multiplied by the body weight, the blood volume, and the percentage of iron in the hemoglobin molecule. An average value of 8.1 per cent was taken as the blood volume of the rat, and 0.335 per cent as the amount of iron in hemoglobin. Then

$$0.081 \times \text{Body weight} \times \text{Hemoglobin level} \times 0.335 \text{ per cent} = \text{Hemoglobin iron.}$$

Subtracting the initial value from that of the final value would then give an expression for the change of iron due to the change of hemoglobin:

$$\text{Final Fe}_{\text{Hb}} - \text{Initial Fe}_{\text{Hb}} = \Delta \text{Fe}_{\text{Hb}}$$

To account for the change in liver iron it was necessary to know approximately the level of iron in the livers of anemic rats, and what percentage of the body weight was represented by the liver. This was accomplished by analyzing livers from 2 rats taken from litters at the time the remaining litter mates were started on therapy. Results were consistent enough to make such a method seem justified. For this reason, average values for total iron and per cent of body weight represented by the liver were employed. The final values, as determined by analysis, less the initial calculated values, expressed the relative change in liver iron.

$$\text{Final liver weight} \times \text{Level of liver iron} - \text{Initial body weight} \times 5.83\% \times 17.5 \mu\text{g/gm.} = \text{Change in liver iron.}$$

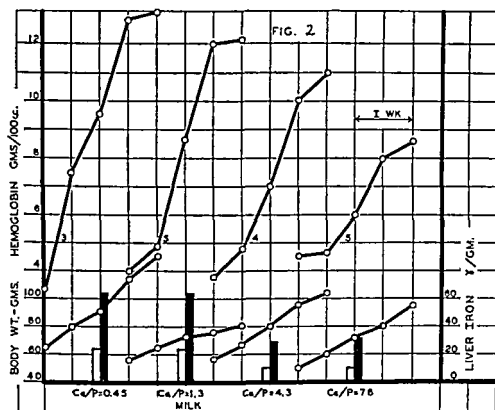


Fig. 2.—Upper curves represent hemoglobin response; lower curves, growth. Bars represent the level of total and available iron in the livers. Iron was fed at a level of 89  $\mu\text{g}$  per gm. and copper at a level of 9.5  $\mu\text{g}$  per gm. Arabic numerals adjacent to the hemoglobin curves indicate the number of animals in the group.

The change in iron due to hemoglobin formation plus the change in iron due to liver storage, divided by the available iron eaten and multiplied by 100 represents the percentage utilized.

$$\frac{\Delta \text{Fe}_{\text{liver}} + \Delta \text{Fe}_{\text{hemoglobin}} \times 100}{\text{Fe}_{\text{(available)}} \times \text{gm. diet eaten}} = \text{Per cent utilized.}$$

Table I summarizes the results obtained when this procedure was applied to the data shown in Figs. 1 and 2 as well as the data from a number of other animals. The results indicate that the utilization of the iron on the diet containing Ca/P=1.87 to Ca/P=2.56 was approximately equal to that on milk. When, however, the calcium-phosphorus ratio was lowered to 0.45,

TABLE I

RAT NO.	SEX	Ca	P	Ca/P	Cu	AVAILABLE Fe	DIET EATEN	Fe EATEN	$\Delta$ Fe $\Delta$ Hb	$\Delta$ FeL	COLON pH	UTILIZED
		%	%		$\mu$ g./gm.	$\mu$ g./gm.	gm.	mg.	mg.	mg.		%
12301	F	0.29	0.648	0.45	9.0	138	130	17.9	3.45	0.301	5.75	20.9
12314	F	0.29	0.648	0.45	9.0	138	152	21.0	4.52	0.226	5.25	22.6
12322	M	0.29	0.648	0.45	9.0	138	167	23.1	4.63	0.215	5.95	21.0
11941	F	0.40	0.649	0.62	9.0	138	122	16.9	3.38	0.369	5.86	22.2
11961	M	0.40	0.649	0.62	9.0	138	92	12.9	2.77	0.212	5.62	23.1
11971	F	0.40	0.649	0.62	9.0	138	125	17.3	3.65	0.489	6.10	23.8
11942	F	1.20	0.64	1.87	9.0	138	108	14.9	2.82	0.121	6.80	19.7
11972	M	1.20	0.64	1.87	9.0	138	145	20.0	3.70	0.378	6.97	20.4
12311	F	1.64	0.64	2.56	9.0	138	178	24.6	4.18	0.228	6.90	17.9
12324	F	1.64	0.64	2.56	9.0	138	156	21.6	3.72	0.101	6.90	17.7
12312	M	1.78	0.404	4.40	9.0	138	147	20.3	3.62	0.136	6.9	18.4
12321	M	1.78	0.404	4.40	9.0	138	166	22.9	3.62	0.150	6.2	16.4
11963	M	1.62	0.404	4.10	9.0	138	92	12.8	2.02	0.107	6.5	14.8
12303	M	3.07	0.402	7.65	9.0	138	147	20.3	2.65	0.124	6.55	13.5
12315	F	3.07	0.402	7.65	9.0	138	138	19.0	1.95	0.066	7.18	10.6
12333	F	3.07	0.402	7.65	9.0	138	125	17.3	1.78	0.076	6.90	10.7
11683	M	Milk		1.3				18.6	3.16	0.246		18.4
11674	F	Milk		1.3				18.0	3.12	0.067		17.7
11691	F	Milk		1.3				12.5	2.14	0.045		17.4

the utilization of iron was markedly increased. There appears considerable latitude in the calcium-phosphorus ratio over which there is little variation in the utilization, especially in the range normally employed in rations ( $\text{Ca/P}=1.3$  to  $4.5$ ).

The effect which the calcium-phosphorus ratio had on iron utilization is shown graphically in Fig. 3. This evidence, in addition to that presented in Figs. 1 and 2 and Table I, establishes the fact that iron utilization decreases with the increase of the calcium-phosphorus ratio and that maximum iron utilization occurs when the calcium-phosphorus ratio is less than that of whole milk.

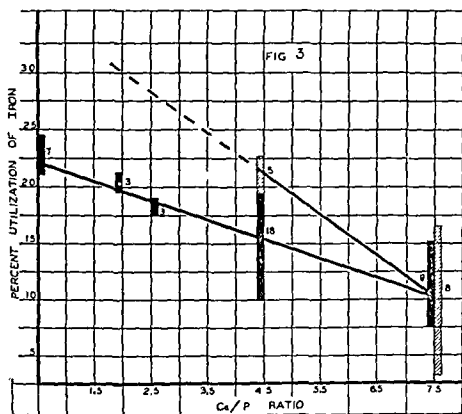


Fig. 3.—Iron level =  $139 \mu\text{g}$  per gm. Solid bars indicate the range of data where  $\text{Cu} = 139 \mu\text{g}$  per gm., lined bars where  $\text{Cu} = 50 \mu\text{g}$  per gm. (in the ration). Adjacent numerals indicate the number of animals from which the data were obtained.

The effect which the calcium-phosphorus ratio has on iron utilization in the presence of additional copper is also shown in Fig. 3. The results indicate that the higher calcium-phosphorus ratios might also be retarding the utilization of copper. The data are insufficient to warrant any conclusions at this time, but work is being continued in this laboratory to check this observation.

The fact that calcium salts in excess make iron salts less available to an animal while phosphates have the opposite effect, indicated the possibility that there might be a difference in the pH of the gastrointestinal tracts of the animals. Results indicated little change in any segment of the tract except that of the colon-cecum segment. As is shown in Table I there was a marked lowering of the pH of the segment on the low calcium-phosphorus ratio. When calcium was added to the ration, the pH was again established between 6.5 and 7.2 which is in agreement with the observations of Robertson<sup>12</sup> who assigns to calcium the role of maintaining the pH of the colon at approximately 7.0.

#### DISCUSSION

The data here presented clearly demonstrate that high levels of calcium carbonate retard iron utilization and that monobasic sodium phosphate has

the opposite effect. That this was utilization and not merely hemoglobin formation was demonstrated by the fact that both hemoglobin regeneration and iron storage in the liver increased as the calcium-phosphorus ratio decreased.

The danger of considering the hemoglobin level as the sole index of iron assimilation is evident when we consider the role copper plays in the regeneration of hemoglobin. Copper does not influence the assimilation of iron but functions in its conversion to hemoglobin. Using the hemoglobin level alone as an index of iron utilization would not be accurate if copper were lacking. Since the formation of hemoglobin is a relatively complex process, there may be many factors which will retard it, among them a high level of phosphorus.

Day and Stein<sup>6</sup> studied the relation of calcium and phosphorus to iron utilization on the low mineral ration of Orten, Smith, and Mendel. They state ". . . in rats on a low mineral ration containing a relative excess of phosphorus, the mild polycythemia and anemia which occur are attributable to the presence of relatively excess phosphorus, and that the effect of calcium in preventing the hematopoietic abnormality resides only in its ability to bind phosphorus, thus permitting the dietary iron to be used for hematopoiesis instead of being excreted presumably as a phosphate."

There seems little doubt that they produced abnormal effects with extreme disturbances of the mineral level. For the most part, they used greater extremes in the calcium-phosphorus ratios than we employed. Our data then are not directly comparable, but in light of the data which we obtained it seems possible that in their experiment the abnormality appeared not as a result of poor iron assimilation but rather as a result of some other effect upon hemoglobin maintenance.

Our results, together with those of other workers reviewed here, seem to indicate that within limits ordinarily reached in most diets, calcium impedes the absorption of iron, while phosphorus has a stimulating effect.

#### SUMMARY

1. A ration is described which, when supplemented with iron and copper, gives equally as rapid growth and hemoglobin regeneration in anemic rats as mineralized milk.
2. Studies on the calcium-phosphorus ratio indicated that a low calcium-phosphorus ratio was favorable to iron utilization since maximum utilization occurred at  $\text{Ca/P} = 0.45$ , with decreasing utilization as the  $\text{Ca/P}$  ratio increased to 7.65.
3. Studies on the pH of the intestinal tract showed that the pH of the colon of rats on a diet with  $\text{Ca/P} = 0.45$  was distinctly lower (5.2 to 6.1) than those on diets with a higher  $\text{Ca/P}$  ratio in which the pH of the colon was 6.2 to 7.2.
4. The stimulating effect obtained with the higher levels of copper indicated that copper is not utilized as completely from such a dry ration as from milk.
5. A method of expressing the percentage of iron utilized by anemic rats during therapy is described.

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## TRANSFUSIONS AND POLYCYTHEMIA IN NORMAL AND TUMOR-BEARING RATS

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ANY of the familiar types of transplantable tumors of rats and mice, situated most conveniently in a leg, may be subjected to asphyxia by temporary ligation of the leg, with two chief results: (a) dark hemorrhagic congestion of the tumor in contrast with the bright reactive hyperemia of the normal tissues after release of the ligature; (b) extensive necrosis of the tumor, sometimes to the point of actual cure, without permanent damage of the normal tissues.<sup>1</sup> As the cause of the tumor death evidently consists in the vascular or inflammatory reaction,<sup>2</sup> inquiry is naturally turned either to intensifying this result or producing it by some other means. One possible factor for consideration is the increased corpuscle content and viscosity of the blood following such ligations.<sup>3</sup> It is conceivable that the desired effects may be assisted if the corpuscle content be still further increased, and perhaps also if the reduced blood volume resulting from ligation be changed to an increased blood volume.

Such a change is opposite to the usual anemia of cancer. The latter, however, is not invariable, for normal blood counts are possible with early cancer and sometimes in the later stages.<sup>4</sup> On the other hand, clinical polycythemia offers some highly suggestive facts, since in the severe form it often leads to stasis and consequent ulceration or gangrene in the extremities or other places of sluggish circulation.<sup>5</sup> Assuming that the makeshift circula-



tion of the tumor stroma constitutes a vulnerable point in the vascular system, it becomes probable that polycythemia will intensify the effects of ligation and may possibly set up a spontaneous process of similar character. Encouraging in this connection is the well-known fact that spontaneous recessions in tumors are often initiated by the occurrence of hemorrhages and infiltrations. These theoretical suggestions are not altogether supported by an inquiry into the possible incompatibility of cancer and polycythemia, because the actual combination is sometimes reported in clinical literature.<sup>6</sup> The rarity of this association, however, still permits the speculation that it exists only with special tumors or under special conditions, or that there may be some degree of true antagonism which may be utilized therapeutically. A rather long and extensive investigation will be reported here only in brief outline, presenting some new observations on both normal and tumor-bearing rats.

#### NORMAL CONTROLS

Rats, weighing between 100 and 200 Gm., will often tolerate transfusions of as much as 10 to 15 c.c. of heparinized blood, or 20 to 25 c.c. may sometimes be given in one day if divided into 2 or 3 injections. A large series of experimental findings may be summarized as follows:

1. Young rats, not fully grown, are sometimes exceptionally sensitive to transfusions and die either immediately or within a short time after receiving as little as 2 or 3 c.c. of blood. No investigation of blood groupings<sup>7</sup> was made. Some special peculiarity seems to exist in these animals, while, on the other hand, the high tolerance of adult rats generally holds good for blood from other strains, including colored rats.

2. Acute death from excessively large transfusions never occurs by heart failure, but always in one of two forms: (a) Apnea. This is adequately explained by the intense pulmonary congestion at autopsy in such cases. But it can also be proved that a needle inserted quickly in the heart or a jugular vein can withdraw more blood than was injected, so as to relieve all plethora and all visible pulmonary engorgement while the heart is still beating, and yet respiration or life is not restored. (b) Liver congestion. This is a remarkable phenomenon, occurring especially in rather young rats. It is evidently not due to any chemical factor but strictly to the quantity of blood. When a certain limit of injection is reached, the liver becomes rather suddenly swollen and tense, so that it is plainly palpable like a hard ball through the abdominal wall. This condition is confirmed at autopsy, when the congestion in the lungs and other viscera is found only moderate, and the spleen (except in one isolated instance) is found noticeably small and free from congestion. Here also the full amount of injected blood, or even more, can be withdrawn by quick aspiration of the heart or a jugular vein while the heart is still beating. The liver swelling and all plethora elsewhere can thus be completely relieved, but death is not prevented. A few trials of epinephrine were also unsuccessful. In either (a) or (b) there is no striking congestion in the brain or spinal cord.

3. After transfusion of too much blood, in either single or divided injections, death occurs after a number of hours or days from pulmonary congestion

or pneumonia. The symptoms are weakness and small quantities of blood-tinged fluid about the nares, evidently indicating pulmonary edema. Only a few hours after an excessive transfusion the lungs may be in a state resembling red hepatization. The slower deaths following smaller or divided transfusions are explained by varying degrees of pulmonary congestion, edema, and pneumonia.

4. Less numerous transfusions were given of separated corpuscles or plasma. Without precise quantitative comparisons, it can only be stated that the animals tolerate a somewhat smaller quantity of corpuscle sediment and a slightly larger quantity of plasma than of whole blood. The ultimate results are similar, except that with corpuscles the congested lungs and other viscera are found very red and with plasma very pale. A similar state of fatal pale pulmonary congestion is also produced by repeated infusions of artificial plasma made with gum acacia.

5. Transfusions of 5 to 10 c.c., repeated at one-, two-, or three-day intervals, are tolerated by different rats for about 4 to 7 times. Death then comes from the lungs as above described. In this way, the most resistant rats can be kept for two to three weeks in a state of plethora with red blood cell counts of 12 to 14 million, with eyes noticeably red and bulging, and with skin perceptibly pink and reddening excessively on slight irritation.

*Ligations of Legs.*—According to the purely mechanical theory of shock, plethoric rats should be able to endure longer ligations than normal animals. The opposite is true, however, since the plethoric animals are liable to die within four or even three hours of ligation of one hind leg, evidently because of subnormal general strength. Congestion in the ligated limb is obviously increased. When the tourniquet is placed low enough to avoid fatal shock, an increased tendency to gangrene seems demonstrable with sufficiently long ligations, but the time limits were not accurately determined.

#### TUMOR EXPERIMENTS

The following is a brief summary of results of over 100 experiments, comprising all three types of rat tumors used (Crocker sarcoma No. 39, Flexner-Jobling carcinoma, and Walker tumor), and single or repeated transfusions, generally of whole blood but of corpuscle sediment or plasma in a smaller number of cases.

Sufficiently large transfusions can of themselves produce extreme congestion in tumors, apparently more marked than in the lungs or any other organs. An important distinction is that this congestion is often followed by extensive necrosis in the tumors, but never in any normal organ. These effects are greatly increased by comparatively short ligations, of one-half to three hours. Such treatment readily produces complete sloughing of the tumor as far as can be judged by gross examination, while the normal leg tissues react with only moderate inflammation. Plasma, whole blood, and corpuscles form an ascending series for the production of all the above changes. Permanently curative results could not be judged because all animals treated sufficiently to cause apparently complete necrosis of tumors died within a few days from shock or pulmonary congestion. Because of the seemingly en-

couraging local results, the experiments were continued through a large series of transfusions as mentioned, also injections of gum arabic and of plain saline or glucose solutions, also with arsenic combinations and other expedients, without escaping the dilemma of death from pulmonary congestion when the transfusions were too large or from shock when the ligations were too long. While these experiments seemed to conform to the theory that a tumor constitutes a vulnerable point in the circulation, the essential difficulty was encountered in the almost equal vulnerability of the lungs.

#### AUTOGENOUS POLYCYTHEMIA

As the fatal consequences of the plethora produced by transfusions in rats are altogether different from the conditions in clinical polycythemia, further series of experiments were performed with the polycythemia produced by administration of metals (iron, copper, cobalt, manganese) as described by several authors.<sup>8</sup> In the successful cases, there was no difficulty in keeping rats for a number of weeks with counts of 12 to 16 million red blood cells without any of the pulmonary or other dangers which occur with transfusions. The principal observations can be summarized as follows:

1. The local and constitutional effects of ligation of legs appear to be somewhat augmented with the autogenous polycythemia as compared with the normal state. No numerical expression of the difference was attempted, but it is far less than with transfusions.

2. Animals with this metal polycythemia, and, on the other hand, animals made more or less anemic<sup>9</sup> by repeated bleedings, showed no significant changes in the number of "takes" after tumor inoculations.

3. As the result of numerous failures from various causes, only 19 rats were finally obtained with the desired combinations of marked polycythemia with a well-established tumor. Ligations for various periods produced distinctly greater hemorrhagic congestion than in normal animals, but this was less than in the transfused animals, and in particular the necrosis was much less. There was perhaps slightly less tolerance for ligation than in normal animals, and any increase in the actual breakdown of tumors was doubtful. No permanent cures were obtained in this comparatively small series.

In general, it must be concluded that polycythemia *per se* does not display any important degree of antagonism to tumor growth. The special congestion and subsequent necrosis of tumors in connection with large transfusions seem to be due to general circulatory failure. It seems doubtful if the desired tumor-killing effect could be obtained in any other species<sup>10</sup> which might stand the transfusions better than rats.

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# DOES HEMOLYTIC STREPTOCOCCUS INFECTION OR SULFANILAMIDE AFFECT THE ALPHA HEMOLYSIN OF SERUM?\*

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CERTAIN observations have been made in the course of routine tests which I feel should be called to the attention of the medical profession and particularly to the notice of those who specialize in serologic and immunologic studies.

In routine blood grouping practice, rapid hemolysis of incompatible cells without agglutination is not commonly encountered. Since Moss' clarifying work,<sup>1</sup> emphasis has been placed on the agglutination. Accordingly, in preparing for transfusions hospital laboratories usually group the donors' cells by observing them in certified strongly agglutinating sera types A, B, and O. Then major and minor cross matchings are made with donors and recipient of the same group. Thus the action of the donors' and recipient's sera on incompatible cells is not ordinarily observed.

Moss<sup>1</sup> wrote in 1910, referring to hemolysis, "In regard to the relationship existing between isoagglutinins and isohemolysins, I may say that agglutination frequently occurs independently of hemolysis but that the inverse relation occurs, i.e., hemolysis without the simultaneous or preceding occurrence of agglutination seems less likely.

"As a rule, however, agglutination proceeds more rapidly than does hemolysis, and by observing the action of a serum, which contains both agglutinin and hemolysin, on susceptible corpuscles, one frequently sees agglutination set in which is subsequently broken up as hemolysis takes place; so that if the observations are not made until the lapse of 2 hours, in a case where the hemolysin is not quite sufficient to dissolve completely all of the corpuscles present, we may get the appearance of hemolysis having taken place without agglutination."

In 1927 J. Buren Sidbury<sup>2</sup> remarked that, "... too little emphasis has been laid on hemolysis in blood matching ... hemolysis is forgotten about in the usual routine of blood matching, since it occurs so infrequently." He observed hemolysis without simultaneous or preceding agglutination 10 times in two years while matching bloods for transfusions.

According to Stitt,<sup>3</sup> agglutinins and hemolysins are separable by heat, and hemolysis is not necessarily preceded by agglutination.

For the convenience of readers the different classifications for the blood groups are tabulated. In the Landsteiner or International classification the

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capital letters represent inherited dominant agglutinogens of cells. Corresponding active agglutinins (or hemolysins) of sera are indicated by the small Greek letters (Bernstein<sup>4</sup>).

Jansky classification	IV	II	III	I
Moss classification	I	II	III	IV
Landsteiner classification	AB	A	B	O
Active agglutinins	—	$\beta$	$\alpha$	$\alpha\beta$

Cell suspensions of customary density were obtained for the blood grouping tests, described herein, by mixing a drop of fresh blood with about 3 c.c. of a solution of 1 per cent sodium citrate in physiologic saline. A drop of the suspension was mixed with a drop of the serum to be tested on a cover glass and inverted over the vaseline-rimmed hollow of a hanging-drop slide. The preparations were rocked back and forth several times to insure mixing before being examined under the microscope. Since all sera were tested before chilling, although some were also tested after being in the refrigerator, we are not here concerned with "cold agglutinins" or "cold hemolysins."

At this point it may be well to describe the degree of hemolysis ordinarily encountered. The effect of 8 sera from known group O individuals on known group A cells from four sources were studied. Agglutination started in all preparations in from one-half to two minutes and was moderate or marked in one and one-half to three minutes. There was no hemolysis in 6, and only the ordinary slow hemolysis in 26 of the 32 preparations. The hemolysis usually began in three to ten minutes, but sometimes not until after one-half hour. It was usually very slow, but in some cases it was moderate. In all preparations the microscopic field was filled with cells after one-half hour and there were many clumps, usually heavy and fused but sometimes loosened. The picture was usually the same after one or one and one-half hours longer, but in a few instances moderate hemolysis dissolved enough of the cells so that there were as few as 40 cells after one and three-quarter hours. The number of cells remaining after a specified time seemed to depend on the density of the cell suspension and the amount of serum used.

When these 8 known group O sera were similarly tested with group AB cells from two sources, agglutination was moderate or marked in two minutes, and slow hemolysis, present in all preparations, began in five to ten minutes. Here again all fields were crowded or moderately filled with cells and showed heavy clumps after one-half hour.

Three instances of very rapid specific hemolytic action with very little or no agglutination by sera of group O patients, who had received sulfanilamide therapy for beta hemolytic streptococcus infection and low grade bacteriemia, are presented.

CASE 1.—A 22-year-old group O male attendant was diagnosed as having a beta-hemolytic streptococcus bacteriemia and treated with very high dosages of sulfanilamide.

Culture of exudate from an infected finger on the day of admission was positive for beta hemolytic streptococci; blood culture showed gram-positive cocci in chains one hour after inoculation of the broth but it was negative after twenty-four hours. Subsequent blood cultures on the seventh and eighth days were negative.

Daily sulfanilamide therapy was begun on the day of admission. The blood level for free sulfanilamide increased from 4.8 mg. per cent on the seventh day to 28.4\* mg. on the eleventh day, and then decreased to 6.7 mg. on the seventeenth day. It was 2.3 mg. on the eighteenth day, at which time it was discontinued.

On the eighth day the blood sugar was 148 mg. per 100 c.c. and the nonprotein nitrogen was 43 mg. per 100 c.c. On the eleventh day the nonprotein nitrogen was 45 mg. per 100 c.c.

The white blood cell count rose from 9,400 per c.mm. on the day of admission to 23,900 on the fourteenth day, and then decreased to 5,500 by the nineteenth day, staying at this level until the thirtieth day when it was 7,600. The red blood cell count decreased from 4,010,000 per c.mm. on the day of admission to 3,280,000 on the fourteenth day and 2,360,000 on the nineteenth day, then it increased steadily to 4,090,000 by the thirtieth day.

The urine showed a slight trace of albumin and a slightest possible trace of sugar on the day of admission. Subsequently no sugar was found. The albumin varied from negative to trace or heavy trace, at which times a rare hyaline or finely granular cast was found in the sediment. A slightly positive foam test for bile was noted on the eighth day only. Urinalyses on the thirtieth and thirty-second days were essentially negative.

No transfusions were given.

The patient was dismissed from the hospital ward on the thirty-first day.

On the eighth day of the patient's illness, while he was receiving sulfanilamide therapy for his streptococcal infection, a preliminary determination of his blood group was made by the method of major and minor cross-matchings, with known group A cells and serum. In five minutes the group A cells which had been mixed with the patient's serum were not to be found. Then it was observed that not only did this serum hemolyze group A cells very rapidly but it did this without any observable agglutination. Under the microscope the individual cells were seen to diffuse, shrink, and disappear in about five minutes. Since, in addition, in the minor matching the group A serum did not agglutinate nor hemolyze the patient's cells, his blood was classified as type O. This grouping was confirmed on the seventeenth day by the non-agglutination of the patient's cells in certified typing sera A, B, and O.

The blood grouping properties were studied further eighty-nine days after the first day of illness which was seventy-one days after discontinuance of sulfanilamide therapy. The patient's serum again rapidly hemolyzed without noticeable agglutination known group A cells from two sources, hemolysis beginning before two minutes and practically complete in five minutes. It similarly hemolyzed known group AB cells from two sources in five minutes. However, Group B cells from two sources were not hemolyzed. Instead the usual rapid agglutination took place, beginning before two minutes and marked in five to six minutes.

Similar blood group characteristics were observed on the ninety-eighth day, eighty days after cessation of sulfanilamide therapy. The patient's cells were again tested in certified sera of types A and B. Since no agglutination nor hemolysis took place during one hour, the blood was again found to belong to group O. The patient's serum still hemolyzed known AB cells from

\*Dr. K. V. Quinn wishes to point out that at one time in this illness, while the patient was receiving the excessively high dosage of 240 grains of sulfanilamide daily for four days, the blood level reached 28.4 mg. for two days. On reducing the dosage, the blood level fell steadily with no unfavorable results except for the 2,360,000 red blood cell count.

two sources and known A cells from two sources without agglutination, the hemolysis beginning in one minute and complete in five minutes, except for one cell left in an originally crowded field. As before there was no hemolysis of known group B cells from two sources even after thirty to forty minutes, the usual agglutination beginning in one minute and marked in two to four minutes. There was no evidence of agglutination or hemolysis of known group O cells from two sources during eighty minutes.

One month later, on the one hundred and twenty-sixth day, about three and one-half months after the sulfanilamide had been discontinued, the hemolytic action was still marked but tended more toward normal. In one to two minutes there was a slight tendency to agglutinate in 2's and 3's group AB cells from two sources and group A cells from four sources, but these clumps were soon loosened and broken up by the fairly rapid hemolysis. This began in about three minutes and finally left in the microscopic field only from 10 to 100 AB cells in twenty minutes and from 0 to 200 A cells in six to twenty minutes. The effectiveness of the hemolysis now seemed to be more dependent on the density of the cell suspension, the proportion of serum, and the source of the cells. As before, the normal agglutination of group B cells from two individuals took place without hemolysis. There was no agglutination or hemolysis of known group O cells from two individuals during one hundred minutes.

CASE 2.—The second patient was a group O female undifferentiated high grade moron, 23 years of age, suffering from a beta hemolytic streptococcal infection of the knee and a low grade bacteriemia treated with sulfanilamide.

Two days after admission blood culture showed gram-positive cocci in pairs. Operation was performed on the seventh day. Cultures from the wound were positive for beta hemolytic streptococci. On the eighth day blood culture was positive for gram-positive cocci in pairs and short chains. Another operation was performed on the twenty-third day. Blood cultures taken on the twenty-third and forty-fifth days were negative.

Sulfanilamide therapy was started on the twenty-fourth day. The blood sulfanilamide level was 5.4 mg. per 100 c.c. on the twenty-fifth day and increased by the thirty-first day to 12.3 mg. where it remained for three days. It fell to 7.4 mg. on the thirty-seventh day, but increased again to 12.5 mg. by the fortieth day. On the forty-first day, when sulfanilamide treatment was discontinued, the blood level was 9.7 mg. per 100 c.c. No sulfanilamide was found in the blood on the forty-fourth day.

The blood sugar was 140 mg. per 100 c.c. on the evening of admission. On the third day the fasting blood sugar was 127 mg. per 100 c.c. On the eighth day the fasting blood sugar was 110 mg. per 100 c.c. and the nonprotein nitrogen was 31 mg. per 100 c.c.

The urine showed a slight trace of albumin and a slightest possible trace of sugar the morning after admission. Thereafter the sugar was negative and the albumin was either negative or slightly positive. On the eighth day an occasional hyaline or coarsely granular cast was found in the sediment and the foam test for bile was slightly positive.

The remaining clinical data seemed irrelevant, and since they will probably be published in another connection, they are omitted here. The patient was still convalescing on the one hundred and second day.

On the twenty-fifth day, one day after sulfanilamide therapy for the streptococcus infection had been started, the patient's blood was typed for transfusion. Since her cells were not clumped in certified sera of groups A, B, and



O, she belonged to the O group. Similarly, her mother and her stepfather were found to belong to the O group. Major and minor cross-matchings of the blood of these donors with that of the patient showed complete compatibility, only harmless, slight but definite rouleau formation being observed in repeated preparations of the patient's serum and the donors' cells. On this twenty-fifth day transfusion was done using the mother's blood with favorable results. Blood from the stepfather was also taken and stored.

As a matter of interest the patient's serum before transfusion was matched with known group A cells. Surprisingly, in this case too there was observed no agglutination but rapid hemolysis complete in a few minutes.

On the thirtieth day the patient's serum was again matched with the cells of the stepfather's preserved citrated blood of five days before, and as before there was only definite rouleau formation. Satisfactory transfusion was done using this blood.

On the forty-fourth day fresh blood from the mother was found to be compatible with the patient's blood and another transfusion was given using this donor.

On the sixty-third day, twenty-two days after the sulfanilamide was discontinued the blood grouping properties were again studied. The patient's serum rapidly hemolyzed known group A cells in seven to ten minutes without apparent agglutination. It also hemolyzed group AB cells in two to seven minutes without agglutination. It agglutinated group B cells in the usual fashion, however, no hemolysis being observed during twenty minutes.

Blood grouping characteristics were observed further on the seventy-third day. As before, the patient's cells were neither clumped nor hemolyzed in certified group A and Group B sera, again indicating that she belonged to the O group. Her serum hemolyzed known group AB cells from two sources and known group A cells from two sources without agglutination, hemolysis starting in one minute and practically complete in four to seven minutes. Again this serum agglutinated known group B cells in the usual fashion, beginning in one to two minutes and becoming marked in three minutes. This time, however, the ordinary slow type of hemolysis of the group B cells was seen to accompany the ordinary agglutination. It began in about two to three minutes and continued so slowly that after one-half hour there were still clumps and about 500 cells in the field; after one and three-fourths hours there were clumps and about 100 cells; after three hours about 15 single cells remained. This was evidently the common type of hemolysis due to the beta hemolysin and very different from the rapid hemolytic action of the group AB and A cells.

One month later, i.e., on the one hundred and second day of illness and two months after the sulfanilamide had been discontinued, the blood group characteristics tended a little more toward normal. Now there was slight to moderate clumping of AB cells from two sources in one to one and one-half minutes, but hemolysis began in two and one-half minutes, disintegrating the clumps and dissolving the cells so that in twenty minutes only 2 to 10 cells

were left in a field originally crowded. Similarly, when the serum was matched with group A cells from four sources there was a very slight to moderate clumping in one-half to one minute, but hemolysis started in one and one-half to two minutes, loosening the clumps and destroying the cells so that in eleven to seventeen minutes only 1 to 5 cells remained in the field. As before there was the usual agglutination of group B cells from two sources, this time with practically no hemolysis. There was neither hemolysis nor agglutination of known group O cells from two individuals.

CASE 3.—A third case is that of a 14-year-old group O female familial low-grade moron. Three days before admission she had been confined to bed with an acute follicular tonsillitis. On the day of admission, the fourth day of her illness, her axilla temperature rose to 103.8° F. and she had severe chest pain. She was diagnosed as having a beginning streptococcus pneumonia and she was treated with sulfanilamide.

Blood culture, taken on the seventh day of illness, was positive after seventy-two hours for rare gram-positive cocci occurring singly and in pairs.

Sulfanilamide therapy was started about 12:01 A.M. of the fifth day and discontinued the thirteenth day. The blood levels were 11.8, 9.9, 12.5, 11.1, 4.7, 6.1, 4.6, 3.2, 1.5, and 0.5 mg. per 100 c.c. from the fifth through the fourteenth days, respectively.

The red blood cell count varied during the illness between 4,260,000 and 5,270,000 per c.mm. The white blood cell count was 13,650 per c.mm. on the fourth day and rose to 17,500 on the eleventh day but decreased to 7,800 by the fourteenth day. The urine was essentially negative except for a very slight trace of albumin.

The patient showed steady improvement under treatment and was discharged on the nineteenth day.

In this case the illness was not so grave and the sulfanilamide therapy was not so long continued as in the previous 2 cases. Nevertheless, a definitely increased rate of hemolysis of group AB and group A cells by this patient's serum was also seen.

Her blood group characteristics were studied on the fifth day of illness and only 15 hours after the first dose of sulfanilamide. The cells, observed during two and one-half hours, did not clump in certified sera types A, B, and O, and the patient, therefore, belonged to group O. Although her serum began a moderate agglutination of known group A cells in one minute, rapid hemolysis started in one to two minutes, disintegrating the clumps and completely hemolyzing in four to five minutes all the cells in a previously crowded field. Similarly, this serum began a moderate agglutination of group AB cells from two sources in one minute, but rapid hemolysis started in one and one-half to two minutes, disintegrating the clumps and completely hemolyzing in four to six minutes all the cells except one in a previously crowded field. Matching with known group B cells from two sources resulted in the usual agglutination, beginning before one minute and marked in two to three minutes, without noticeable hemolysis.

Four days later, on the ninth day of illness and after five days of sulfanilamide, her serum showed a slight agglutination of AB cells in one-half minute but hemolysis began in one to two minutes disintegrating the clumps and dissolving the cells so that in six to fourteen minutes there were only 1 to 20 cells left in the microscopic field. The number of AB cells remaining ap-

parently depended somewhat on the density of the cell suspension and the amount of mixing motion given to the preparations. This serum also started to agglutinate known group A cells from one individual in one to one and one-half minutes but rapid hemolysis set in in one and one-half minutes, disintegrating the clumps and dissolving the cells so that after six to fifteen minutes only 1 to 7 cells remained in the field. There was only a very slight tendency to clump A cells from another individual, rapid hemolysis dissolving all the cells in three minutes. The action on group B cells from two sources was the usual agglutination without hemolysis. On group O cells from two sources there was no effect except for slight rouleau formation.

On the twenty-second day, nine days after discontinuance of the sulfanilamide, the hemolysis was still somewhat rapid. The serum began a slight to moderate clumping of AB cells from two sources in one-half minute, but hemolysis started in two minutes, broke up the clumps, and left only 2 to 7 cells in the field after nine to fifteen minutes. However, with an exceptionally heavy cell suspension, about 50 cells remained, showing that the hemolysis was neither so strong nor so rapid as to be independent of the number of AB cells. The action of this serum on known A cells was similar. In one-half to one minute there began a slight tendency to clumping which became more marked in the succeeding minute. However, rapid hemolysis set in in about two minutes, breaking up the clumps and dissolving the A cells so that in six to thirteen minutes only 1 to 6 cells remained in previously crowded fields. As before, group B cells from two sources were clumped at once without evidence of hemolysis. There was neither agglutination nor hemolysis of group O cells from two sources during one and one-half hours.

#### DISCUSSION

This rapid hemolysis by sera of group O patients having beta hemolytic streptococcus infection treated with sulfanilamide is evidently very different in degree at least from the ordinary two-hour hemolysis mentioned by Moss. Inasmuch as it is specific for cells containing the A agglutinin, since only group AB and group A cells are thus hemolyzed, it is probably associated with the alpha agglutinin of the serum. In this connection it is of interest that the serum of a group A patient who had been treated with sulfanilamide showed no unusual blood grouping properties. This is consistent with the foregoing, since the alpha agglutinin in group A serum is inactive.

Interpretation is, of course, impossible with only 2 or 3 cases. Unfortunately, blood grouping tests were not made before the administration of sulfanilamide. However, the definite though temporary agglutination preceding the somewhat less rapid and less complete hemolysis, which was observed in the third case and also in the other two cases two or three months after cessation of sulfanilamide therapy, suggests that the rapid hemolytic action is probably not normal for these individuals. Moreover, it is probably not due to the infection alone, since then the phenomenon would have been noticed long ago. A glance through recent literature revealed no mention

of such increased group-specific hemolytic action of serum due to infection. Wünsche<sup>5</sup> did not mention it in a paper on the relation between the blood group and the hemolytic power of streptococci. Therefore, it seems more logical to assume that the effect is due to the action of sulfanilamide on the alpha hemolysin.

For some time workers have sensed that there might be some connection between the blood groups and susceptibility or immunity to disease. Alexander<sup>6</sup> thought that the tendency to malignant disease depended on the blood group, but this was denied.<sup>7</sup>

A few published observations point to an immunologic peculiarity of the alpha agglutinin. Straszyński<sup>8</sup> in 1925 found that the Wassermann reaction was related to the blood group, group AB having 2.4 times as many positive Wassermann reactions as group O. He concluded that the rapidity with which the Wassermann reaction disappears under the influence of anti-syphilitic treatment is a constitutional property existing in correlation with the blood group.

Hirszfeld, Hirszfeld, and Brokman<sup>9</sup> found that the susceptibility to diphtheria, as indicated by the Schick test, is inherited conjointly with the blood properties and must also depend on constitutional characters. They stated that infection or immunization can mobilize unrevealed immunologic forces and can accelerate the process of changing from Schick positive to Schick negative, but that this is dependent on the constitutional inherited capacity of the individual.

Balgairies and Christiaens<sup>10, 11</sup> found a remarkable elevation of agglutinin anti A after injection of diphtheria antitoxin and of tetanus antitoxin, which diminished progressively to a stable level in six months and which they attributed to the presence in the antitoxin of a substance having the same nature as agglutininogen A.

An analogous explanation for the cases described herein would be that sulfanilamide is related to agglutininogen A and that in the body it stimulates formation of the anti A or alpha hemolysin. However, the action may be more indirect through an influence on the liver, kidney, or bone marrow, etc.; or, since with physiologic saline there is said to be potentiation<sup>12</sup> of the bactericidal effect of this drug, perhaps its action is catalytic. It may thus upset a balance of hemolytic-agglutinative activity in favor of the hemolysis. In this connection it is of interest that in Cases 1 and 2, when hemolysis without agglutination was substituted for agglutination in the scheme for determining the blood group, the result agreed with that found when only the cells were tested in certified sera. This supports a theory of the interdependent action of hemolysin and agglutinin.

Further study of the facts relating to this hemolysis may eventually lead to interesting practical and theoretical conclusions. For instance, such a study may help us to understand and to prevent certain unfavorable transfusion reactions; it may explain the curative action of sulfanilamide as well as its now unpredictable toxicity for some patients and its inefficacy for others; it may give us a clearer picture of the mechanisms underlying the

action not only of sulfanilamide and perhaps of antisyphilitics and other chemicals, but even of immunizing preparations. However, the aim of this paper is neither to add to the list<sup>13</sup> of conjectures as to the mode of action of sulfanilamide nor to interpret the findings presented. It is merely intended (1) to record the facts of rapid group-specific (anti A) hemolysis with little or no agglutination by sera of three group O patients having beta hemolytic streptococcus infection treated with sulfanilamide; (2) to point out the possible theoretical and practical importance of these facts; and (3) to request that physicians in general hospitals will likewise report the effect of sera of their patients, especially of group B and group O patients, on cells of the different groups before, during, and after sulfanilamide therapy.

I am grateful to the laboratory and medical staffs of the Wrentham State School for their advice and assistance.

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## VITAMIN A DEFICIENCY IN DISEASES OF THE LIVER: ITS DETECTION BY DARK-ADAPTATION METHOD\*

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THERE is considerable evidence that the liver plays an important role in regulating the concentration of vitamin A throughout the body. We are indebted to Moore<sup>1</sup> for stressing this point and for demonstrating the liver to be the chief depot for vitamin A storage in man. This has also been shown experimentally by Sherman and Boynton.<sup>2</sup>

The experimental work of Simonnet and Bousson<sup>3</sup> and Oleott and McCann<sup>4</sup> would indicate that the conversion of carotene, the precursor of vitamin A, is effected in the liver, presumably, by an enzyme called carotenase. However, this conversion in man is not essential when vitamin A is provided in the diet.

That the metabolism of vitamin A is linked with the hepatic system may be illustrated by the occurrence of signs of avitaminosis A in some patients with prolonged jaundice, despite the fact that their diets are quite adequate in vitamin A content. Thus, Altschule<sup>5</sup> found microscopic evidence of vitamin A deficiency in 6 out of 11 patients suffering with severe and protracted jaundice due to congenital atresia of bile ducts. The association between hepatic disease with jaundice and vitamin A deficiency is further illustrated by the observations of Blackfan and Wolbach<sup>6</sup> and Owen and Hennessey.<sup>7</sup> They found epithelial metaplasia in certain tissues in spite of the fact that the patients received an amount of vitamin A in their diets considered sufficient for the protective needs of the body. It is probable that the manifestations of avitaminosis A were conditioned by defective utilization, storage, or absorption of vitamin A, or its precursor, carotene.

When the parenchyma of the liver is injured, a disturbance in its function is to be expected. Early clinical features of vitamin A deficiency in such pathologic states may be overlooked; they occur irregularly and inconstantly, before definite ocular changes develop. The eye changes (xerophthalmia, etc.), as is generally held, occur in the advanced stages of the disease.

The early recognition of vitamin A deficiency in the course of liver disease assumes practical importance, particularly since parenteral administration of vitamin A has become available recently. The liver is known to be instrumental in the formation and storage of the other vitamins, particularly B<sub>1</sub> and B<sub>2</sub> complex, and vitamins C and D. It is also claimed that the presence of vitamin A in the liver enhances glycogen storage.<sup>8</sup>

The purpose of this investigation was to determine the early occurrence of avitaminosis A in patients with hepatic disease, measured by dark adaptation

\*From the Philadelphia General Hospital and the Temple University Hospital.  
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TABLE I  
SUMMARY OF FINDINGS IN CASES WITH LIVER DISEASE

NO.	NAME	AGE	SEX	DURATION OF DISEASE	DARK-ADAPTATION THRESHOLD IN MICRO-PHOTONS	DIAGNOSIS	SIGNIFICANT LABORATORY FINDINGS	REMARKS
1	W. A.	64	M	2 years	1,691	Laënnec cirrhosis	Sugar, 91; urea N, 9; cholest., 90; ester, 50; tot. pro., 6.5; alb., 1.8; glob., 4.7; ict. index, 10; Kahn, neg.	Paracentesis—5 liters and 12 oz. of clear, yellowish fluid removed. Liver palpable halfway to umbilicus
2	R. B.	42	M	10 days	636	Hepatocellular jaundice	Sugar, 70; urea N, 10; cholest., 144; ester, 19%; tot. pro., 4.7; alb., 3.2; glob., 2.1; ict. index, 125. Bromsul, 37% retention. Takata-Ara strongly pos.	Three days after receiving neosalvarsan developed jaundice and epigastric pain
3	S. B.	43	M	14 months	546	Syphilitic cirrhosis	Sugar, 61; urea N, 16; cholest., 148; ester, 65 (38%); ict. index, 13. Wass. ++	Exploratory laparotomy done. Syphilitic cirrhosis (hepar. lobatum)
4	H. B.	48	M	18 months	546	Laënnec cirrhosis	Sugar, 107; urea N, 12; cholest., 142; ester, 51%; tot. pro., 5.7; alb., 2.1; glob., 3.6; ict. index, 20. Takata-Ara strongly pos. Bromsul, 25% ret.	Patient heavy drinker. Seventeen months ago had Talma operation. Felt well until present admission. Twelve paracenteses. Patient died with hemorrhage from bowel
5	R. C.	23	M	1 month	Normal	Hepatocellular jaundice	Sugar, 107; urea N, 10; N.P.N., 36; cholest., 141; ester, 81 (55%); tot. pro., 8.3; alb., 4.5; glob., 3.8. Takata-Ara pos. 1ct. index, 23; Wass. neg.	Jaundice developed during stay in hospital. Previous history of gall bladder. Jaundice cleared on discharge from hospital

6	W. G.	25	M	1 month	1,025	Syphilitic hepatitis	Sugar, 72; urea N, 9; cholest., 320; ester, 87 (22%); tot. pro., 7.1; alb., 3.7; glob., 3.4; ict. index, 72. Takata-Ara neg; phosphatase, 10; Wass. ++	Liver 10 cm. below costal margin. Iodides caused decrease in size of liver to 3 cm. Jaundice cleared
7	W. G.	37	M	3 weeks	Normal	Hepatocellular jaundice (catarrhal)	Sugar, 87; urea N, 11; ict. index, 20. Wass. neg.	Jaundice following attack of pneumonia
8	J. B.	50	M	5 years	19,600	Syphilitic cirrhosis of the liver	Sugar, 63; urea N, 22; cholest., 192; ester, 91 (47%); tot. pro., 7.5; alb., 3.2; glob., 4.3; ict. index, 10. Bromsul., 24% retention. Wass. ++	Numerous paracenteses. Antisyphilitic treatment, 2 c.c. bismuth and mercupurin. Ascites under control 3 weeks prior to admission to hospital when ascites recurred; 2½ liters clear yellow fluid obtained. Liver edge 5-6 cm. below costal border. No recurrence of ascites. Is under antisyphilitic treatment. Has also aortic valvulitis
9	V. D.	48	M	2 months	1,007	Atrophic cirrhosis	Sugar, 103; urea N, 13; cholest., 156; ester, 53%; tot. pro., 6.5; alb., 2.3; glob., 4.4; Takata-Ara strongly positive. Wass. neg.	Patracentesis yielded 5½ liters of yellow fluid. Liver normal size. Spleen palpable to umbilicus. Drank 4-5 glasses wine daily for several years
10	W. K.	56	M	3 weeks	*1,190 1,130 1,434	Atrophic cirrhosis of the liver	Sugar, 65; cholest., 78; ester, 54%; tot. pro., 6.0; alb., 2.1; glob., 3.9. Bromsul., 28% retention	Consumed a pint of whiskey daily for 5 months. Vomited blood. Distended abdominal veins. Ascites. Four months later patient died of pneumonia
11	M. L.	46	F	1 year	309	Carcinoma of the gall bladder with metastasis to the liver	Sug., 81; urea N, 9; cholest., 309; tot. pro., 6.9; alb., 4.2; glob., 2.7; Takata-Ara strongly pos.; ict. index, 85	Exploratory laparotomy revealed carcinoma of gall bladder with metastasis to the liver
12	A. M.	20	F	14 days	408	Hepatocellular jaundice following neosalvarsan	Sugar, 59; urea N, 8; cholest., 176; ester, 81 (46%); ict. index, 42. Wass. ++	Patient delivered of healthy baby. Had granuloma inguinale. Given tartar emetic every 2 days in increasing amounts. On 3rd day received 5 c.c. After receiving neosalvarsan and tartar emetic, she developed jaundice and fever. General condition improved on discharge from hospital, but still jaundiced

\*The thirty-minute thresholds taken at different times.



TABLE I—CONT'D

NO.	NAME	AGE	SEX	DURATION OF DISEASE	DARK-ADAPTATION THRESHOLD IN MICRO-PHOTONS	DIAGNOSIS	SIGNIFICANT LABORATORY FINDINGS	REMARKS
13	R. M.	62	F	2 years	204	Atrophic cirrhosis with jaundice	Choles., 276; ester, 40%; ict. index, 34	Patient consumed ½ pint of whiskey daily for 25 years. Liver 4 fingerbreadths below costal margin
14	R. M.	35	M	3 months	Normal	Atrophic cirrhosis	Choles., 170; ester, 102 (54%); tot. pro., 5.4; alb., 2.3; glob., 3.1; ict. index, 9. Blood vitamin C 0.36 mg.	Two paracenteses. Liver 5 cm. below costal margin. Heavy drinker
15	P. R.	54	M	4 months	273	Cirrhosis of the liver	Sugar, 96; urea N, 26; choles., 108; ester, 66 (61%); tot. pro., 5.2; alb., 2.0; glob., 3.0; ict. index, 8	Paracentesis yielded 2½ gallons of clear yellowish fluid
16	J. D.	44	M	8 months	233	Primary carcinoma of the liver	Sugar, 100; urea N, 12; tot. pro., 5.8; alb., 2.9; glob., 2.9; choles., 224; ester, 120 (53%). Bromsul., 8% retention	Marked ascites. Liver hard and nodular
17	H. D.	17	F	2 months	Normal	Acute catarrhal jaundice	Sugar, 98; urea N, 8; choles., 126; ester, 63 (50%); ict. index, 60	Left hospital free from symptoms except slight jaundice which is clearing
18	Z. H.	33	M	2 months	*572 694 744 1,309	Hepatocellular jaundice	Sugar, 68; urea N, 9; ester, 50; tot. pro., 6.1; choles., 504; alb., 2.8; glob., 3.3; ict. index, 110. Takara-ara strongly pos.	Jaundice following injections of neosulvarsan. Cholesterol kept rising to 1,490 mg. with 2% ester.

19	E. M.	54	M	2 months	240	Cirrhosis following hepatocellular jaundice	Sugar, 79; urea N, 12; cholest., 194; tot. pro., 7; alb., 3.9; glob., 3.1; ester, 105 (53%). Takata-Ara strongly pos. Bromsul., 24% ret.; ict. index, 12	Jaundice cleared after 9 weeks' hospitalization. Liver 3 cm. below right costal margin with tenderness in the right upper quadrant
20	E. G.	29	F	10 years	299	Biliary cirrhosis	Sugar, 6.8; urea N, 10; cholest., 370; ester, 192 (52%); tot. pro., 6.8; alb., 3.9; glob., 2.9; bromsul., 40% retention; ict. index, 43. Takata-Ara neg.	Tenderness and rigidity in right upper quadrant. Liver and spleen enlarged and tender. Poorly functioning gall bladder (x-ray). First gall bladder episode 10 years ago. Recurrent attacks of jaundice with gastrointestinal symptoms
21	G. R.	53	M	3 weeks	390	Laënnec cirrhosis	Sugar fasting, 85; cholest., 300; ester, 56%; tot. pro., 6.5; alb., 2.9; glob., 3.6. Bromsul., 40% retention. Ict. index, 115; Wass. negative. Phosphatase, 19 units	Patient drank couple of beers and whiskeys daily since 1909. Liver edge felt 5 fingerbreadths below right costal edge. Edge smooth and movable. Three paracenteses averaging 3,600 c.c. Patient died in hepatic coma
22	A. C.	68	M	11 days	285	Hepatocellular jaundice	Sugar, 98; cholest., 218; ester, 108; urea N, 17; ict. index, 46. Wass. neg.	Jaundice following acute sinusitis. Patient remained in hospital 18 days. Jaundice disappeared when he left hospital
23	D. S.	70	M	2 years	28,625	Carcinoma of the liver	Ict. index, 97; blood Wass., +4. Van den Bergh direct strongly pos.	Diagnosis of primary carcinoma of liver confirmed by post-mortem examination of tissue
24	W. C.	58	M	3 years	19,670	Primary carcinoma of the liver	Blood sugar, 75; urea N, 18; cholest., 240; ict. index, 17; Wass. neg.	Paracentesis—2,300 c.c. bloody fluid obtained. Diagnosis confirmed by post-mortem examination. Ascitic fluid 300 c.c. on 2/20/39, 3 days before death
25	A. R.	72	M	2 months	177	Calculus in the common duct	Blood sugar, 87; urea N, 12; ict. index, 80	Jaundice progressively worse for past 2 months. X-ray of stomach and intestines negative

under controlled conditions. We selected the dark-adaptation method because evidence has been presented by various investigators that poor dark adaptation is directly related to vitamin A deficiency.<sup>9</sup>

The effect of vitamin A therapy on the progress of the illness, as well as on the hepatic function, is under investigation and will be described elsewhere.

*Dark Adaptation.*—"Dark adaptation" is the ability of the eye to adapt itself to darkness. An example of this is the entering into a darkened cinema theater after walking in the street on a sunny day. At first it is impossible to see the seats in the theater due to the fact that the visual purple was bleached out by the sun. After a specific interval, the objects and people within the theater appear somewhat clearer. As time goes on, this improvement in vision progresses until the vision becomes as clear as if the theater were illuminated. This physiologic phenomenon is accomplished through the regeneration of the visual purple and depends, in the main, upon its vitamin A content.

Pathologic dark adaptation is divided<sup>10</sup> into two types: (1) organic and (2) functional. The *organic* type is the result of deep retinal or choroidal pathology and is recognized by ophthalmoscopic examination. It is noted in such ocular diseases as retinitis pigmentosa, choroiditis, etc. In the absence of retinal, choroidal, or other ocular pathology, a delay in regeneration of visual purple above the normal and specified time is called a *functional or latent* pathologic dark adaptation. This latter condition is attributed to an avitaminosis A.

*Technique.*—All tests were carried out in a darkroom by the method described elsewhere,<sup>11</sup> with the American Optical Co. instrument devised by one of us (F.).

*Material.*—This presentation is based on a study of 25 persons. Careful history, physical examination, and ophthalmologic and laboratory studies were made on each patient. The patients were unselected and represented various types of liver disease as studied in the wards of the Philadelphia General Hospital and the Temple University Hospital during 1938 and 1939.

## RESULTS

A summary of the significant findings in these patients is given in Table I. Thirteen patients with cirrhosis of the liver were examined. Six patients in this group gave a history of alcoholism, and 3 with a plus 4 Wassermann had a history of syphilis.

Patient E. G. in Table I represents the only case of biliary cirrhosis in our series. Her hepatic symptoms were of ten years' duration and were secondary to a chronic cholecystitis. The liver function tests showed moderate hepatic damage.

In patients with Laënnec cirrhosis, the livers were hard and palpable, with ascites in all cases. The liver function tests, as shown in Table I, column 7, displayed varying degrees of liver damage. Jaundice was absent in 4 patients. Seven patients showed jaundice, varying from a slight icteric tinge of the sclerae (icterus index 13) to a diffuse jaundice of the body (icterus index 115). In 2 patients the icterus index was not determined.

While it is fully realized that an absolute diagnosis of cirrhosis of the liver can be made during life only by biopsy, in this group the history and the

course of the disease, coupled with the laboratory studies and clinical data, aided in confirming the diagnosis. In 4 cases the diagnosis was corroborated by autopsy.

All patients with cirrhosis of the liver, including the syphilitic type, showed degrees of pathologic dark adaptation, varying from 204 to 19,600 microphoton.\* Three patients complained of night blindness, determined after careful questioning.

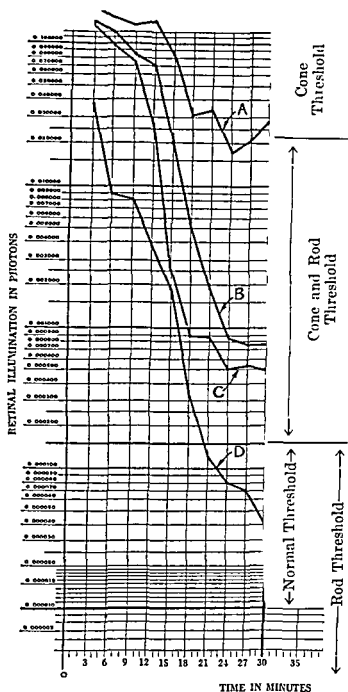


Fig. 1.—Illustrating various types of dark adaptation curves in a series of cases. A. Case 23, Table I, carcinoma of the liver. B. Case 1, Table II, delirium tremens and neuritis. C. Case 4, Table I, hepatocellular jaundice (neocarsphenamine). D. Case 17, Table I, hepatocellular jaundice (catarrhal).

It is interesting in this connection to note that Patek<sup>12</sup> and Snell<sup>13</sup> have reported clinical improvement in individuals with cirrhosis of the liver following the conventional treatment combined with vitamin therapy.

Pertinent to this discussion is the recent study by Connor,<sup>14</sup> in which he maintains that it is very doubtful whether a person consuming a balanced diet in addition to an excessive amount of alcohol would develop alcoholic cirrhosis.

\*Normal dark-adaptation threshold is up to 150 microphotons within thirty minutes.

TABLE II  
SIGNIFICANT FINDINGS IN ALCOHOLIC ADDICTS

NO.	NAME	AGE	SEX	DURATION OF DISEASE	DARK-ADAPTATION THRESHOLD IN MICRO-PHOTONS	DIAGNOSIS	LABORATORY FINDINGS	REMARKS
1	F. B.	26	M	4 weeks	766	Incipient delirium tremens and neuritis of lower extremities	Blood sugar 87. Urea N 11. Kahn negative	Addicted to alcohol since 1931. Consumed 1 quart alcohol daily for 4 weeks. Had no other food but soup and tea. Liver and spleen not palpable
2	T. M.	59	M		701	Alcoholic psychosis and cerebral arteriosclerosis	Blood sugar 68. Urea N 10. Kahn negative	Chronic alcoholic. Lives alone. Drinks 1 or 2 jiggers of whiskey a day and some beer. Liver and spleen not palpable. Diet soup and bread
3	F. C.	35	M	Several weeks	292	Delirium tremens (sore mouth and tongue)	Blood sugar 63. Urea N 11. Kahn negative	On admission patient presented sore mouth and tongue. He was on a high vitamin diet for 19 days prior to dark adaptation
4	F. D.	23	M	1 week	110	Delirium tremens	Blood sugar 82. Urea N 13. B.M.R. -24%. Wassermann negative	Addicted to alcohol for 5 years. For 1 week prior to admission has eaten 2 bowls of soup and 2 cups of tea daily. Liver and spleen not palpable. Has colloid goiter. Patient was receiving desiccated thyroid, 1 grain daily. Is night blind
5	M. L.	42	M	Several weeks	196	Incipient delirium tremens	Blood sugar 87. Urea N 8. Kahn negative	Addicted to alcohol for 5 years. Liver and spleen not palpable
6	J. D.	34	M	4 months	339	Alcoholic psychosis	Blood sugar 79. Urea N 13	Addicted to alcohol for 9 years. Liver and spleen not palpable. Diet soup and bread
7	F. W.	46	M		204	Involutional state Chronic alcoholism	Blood sugar 82. Urea N 13. Wassermann negative	Dark adaptation 2 months after admission. Was receiving betaxin for 2 weeks prior to dark adaptation. Liver and spleen not palpable
8	P. M.	40	M		425	Alcoholic hallucination	Blood sugar 68. Urea N 9	Chronic alcoholic addict. Has been getting betaxin for 1 month prior to dark adaptation
9	P. O.	55	M	3 months	251	Alcoholic neuritis	Blood sugar 89. Urea N 9. Wassermann and Kahn negative	Addicted to alcohol for several years. Takes 3 drinks daily. Food consists of meal, bread, calves' liver, one-half ounce weekly, milk one glass daily, spinach, carrots and potatoes in small amounts
10	H. F.	49	M	Few days	100	Bromide psychosis Chronic alcoholic	Blood sugar 68. Urea N 10. Blood bromide 160. Wassermann negative	Chronic alcoholic. Has consumed a pint of whiskey daily for several days prior to admission. A few days prior to admission he had been taking bromide and the epinephrine treatment.

In 10 patients addicted to alcoholism who were admitted to the psychopathic ward of the Philadelphia General Hospital, a study of their dietary regime prior to admission revealed a very poorly balanced diet, especially inadequate in vitamin A and vitamin B content. Dark-adaptation studies in this group showed that all except one had a marked pathologic curve (see Table II).

There were 7 patients with hepatocellular jaundice (see Table I), 3 of whom apparently utilized and stored their vitamin A, as evidenced by the normal rate of their regeneration of visual purple. The dark adaptation readings of these patients were, respectively, 42, 83, and 130 microphotons (normal up to 150 microphotons). Clinically these 3 patients manifested a minimal degree of liver damage, as evidenced by the clinical course of their illness and the liver function tests. Before their discharge from the hospital, the jaundice had entirely disappeared. A fourth patient belonging to this group had a slightly pathologic dark adaptation. When he left the hospital, his jaundice was clearing.

It is quite significant that in the remaining 3 patients there was marked pathologic dark adaptation: 408, 572, and 636 microphotons, respectively.\* In each case the jaundice followed the administration of neoarsphenamine. The degree of jaundice was of the severe toxic type. Their clinical course was very stormy, and their various laboratory findings showed severe liver damage. When they were discharged from the hospital, there was still some evidence of jaundice. It is worthy of note that the dark adaptation pathology appeared to run parallel with the degree of liver damage conditioning the jaundice as gauged by the icterus index and other liver function tests. Thus, for example:

Case A. M., icterus index was 42, had a dark adaptation threshold of 408.

Case Z. H., icterus index was 110, had a dark adaptation threshold of 572.

Case R. B., icterus index was 125, had a dark adaptation threshold of 636.

We examined 4 patients with carcinoma of the liver, all of whom died. Their diagnosis was confirmed by the histologic examination of the tissues. They all presented pathologic dark adaptation to a marked degree (see Fig. 1, A).

One patient with advanced jaundice had a normal dark adaptation. The jaundice in this case was caused by a silent stone obstructing the common bile duct.

#### SUMMARY AND CONCLUSIONS

Twenty-five patients with various types of liver disease have been studied.

Ten patients with alcoholism, without clinical evidence of liver disease, were also examined.

Complete clinical laboratory and dark adaptation studies were made in each individual case.

\*The light threshold in dark adaptation is the minimum light just visible to the dark adapted eye. Usually threshold readings are taken at three-minute intervals. Where the single threshold is given, it is the last or thirty-minute threshold, which in the normal case should be 150 microphotons.

A photon is a unit of retinal illumination and equals one candle per square meter per square millimeter of pupil area. A microphoton is one-millionth of a photon.

In 21 patients with liver disease the dark adaptation was pathologic. These patients, clinically and by laboratory studies, showed varying degrees of liver damage.

Of 7 patients with hepatocellular jaundice, 3 presented normal dark adaptation and minimal degree of liver damage. The remaining 4 patients showed evidence of liver damage and pathologic dark adaptation. In these 4 cases, the degree of liver damage, as evidenced by the icterus index and other laboratory studies, appeared to run parallel with the degree of dark adaptation pathology.

Nine out of 10 alcoholic addicts showed vitamin A deficiency as revealed by dark adaptation pathology. The tenth person, whose dark adaptation was normal, was receiving desiccated thyroid at the time the test was made.

We are indebted to Dr. M. M. Pearson for cooperation in the study of the alcohol addicts.

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1727 PINE STREET

# THE SOURCES OF THE ENZYMES OF NORMAL AND PATHOLOGIC CEREBROSPINAL FLUID\*

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## INTRODUCTION

IN A PREVIOUS study<sup>1</sup> we found that normal cerebrospinal fluid contains small amounts of beta-glycerophosphatase and amylase, and occasional traces of lipase. In pathologic conditions of the central nervous system, such as purulent and tuberculous meningitis, hydrocephalus, brain tumor, brain abscess, and brain cyst, the cerebrospinal fluid was found to have increased enzymatic activity. The fluid of tuberculous meningitis was characterized by the presence of trypsin, antitrypsin, and tributyrinase, and by increased phosphatase, lipase, and amylase activity. The fluid of purulent meningitis was similar to that of tuberculous meningitis, except that trypsin and phosphatase were found in much greater amounts in the former. The cerebrospinal fluid of hydrocephalus, brain tumor, abscess, and cyst was found to have high tributyrinase and lipase activity. Antitrypsin and increased phosphatase were also found in some fluids of the brain lesion group.

Early in the course of our work the question of the source of the cerebrospinal fluid enzyme arose. We suggested, on theoretical grounds, that the enzymes of normal cerebrospinal fluid may result from the metabolism of brain tissue or may be derived from the enzymes of the blood plasma. The increased enzymatic activity of pathologic cerebrospinal fluid may be due to: (a) pleocytosis of the cerebrospinal fluid, (b) increased permeability of the meninges to plasma enzymes, and (c) destruction of brain tissue. We shall attempt here a discussion of the sources of the cerebrospinal fluid enzymes on a more experimental basis.

## I. THE ENZYMES OF NORMAL CEREBROSPINAL FLUID

The question of the source of the enzymes of normal cerebrospinal fluid must be answered in terms of the source of the cerebrospinal fluid itself, more particularly in terms of the source of the proteins and cells of the cerebrospinal fluid. The latter, however, is still unsettled. According to Dandy and Blackfan,<sup>2</sup> Weed,<sup>3</sup> and Cushing,<sup>4</sup> the fluid is derived from the choroid plexus within the ventricles, and is absorbed from the subarachnoid space chiefly into the cerebral sinuses. Merritt and Fremont-Smith,<sup>5</sup> as a result of their chemical studies, be-

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lieve with Mestrezat that the cerebrospinal fluid is best understood if it is looked upon as a dialysate of the blood plasma. On the other hand, Hassin<sup>7</sup> maintains, on the basis of histopathologic studies, that the cerebrospinal fluid consists of tissue fluids which pass out from the neural tissue along the perivascular spaces into the subarachnoid space and perhaps the ventricles. At present it is generally considered, according to Flexner,<sup>8</sup> that “. . . the cerebrospinal fluid probably arises in large part from the highly vascular choroid plexuses. To fluid from this source is added a relatively small, perhaps insignificant, amount of fluid from the ependymal cells of the ventricles. The ventricular fluid from these origins slowly flows into the subarachnoid space where it receives an addition, unknown in quantity, but probably small, from the perivascular spaces of the brain, and, in the opinion of some, from the blood vessels traversing the subarachnoid space.”

It is impossible to determine from the foregoing data whether the blood plasma or the neural tissue is the more important source of the cerebrospinal fluid enzymes. According to Boyd,<sup>9</sup> the lymphocytes of normal cerebrospinal fluid appear to pass from the perivascular spaces into the subarachnoid space. This, together with the fact that lumbar and cisternal fluids are richer in protein and cells than is ventricular fluid, makes it seem likely that a considerable portion of the enzymes comes from the neural tissues. Whether this portion is greater than that coming from the choroid plexuses and the blood vessels traversing the subarachnoid space seems impossible to settle at present because of our inability to study the enzymes of the neural tissue fluid.

## II. ENZYMES OF THE CEREBROSPINAL FLUID IN MENINGITIS

*A. Leucocytic Enzymes.*—It was noticed that the tryptic activity of the cerebrospinal fluid of purulent meningitis was related to the polymorphonuclear leucocyte content of the fluid.<sup>1, 10</sup> Although no exact correlation was possible, fluids with high polymorphonuclear content generally had greater tryptic activity than fluids with low polymorphonuclear content. In order to test the effect of the presence of polymorphonuclear cells, a sample of lumbar fluid from a patient with streptococcic (hemolytic) meningitis was divided into two parts, one of which was filtered through a Seitz filter. The tryptic activity of the original untreated fluid and of the filtrate were then determined.\* The results, together with those obtained with a second sample of fluid from the same patient,

TABLE I

PATIENT	PORTION STUDIED	DIAGNOSIS	DESCRIPTION OF FLUID	CELL COUNT	TRYPSIN (UNITS)
1. J. D.	(a) Original fluid	Streptococcic meningitis	Lumbar, turbid	2,290	0.53*
	(b) After Seitz filtration		Clear	0	0.0
2. J. D.	(a) Original fluid	Streptococcic meningitis	Lumbar, opalescent	440	0.20
	(b) After Seitz filtration		Clear	0	0.0

\*Values of this order of magnitude represent relatively high tryptic activity.

\*The methods used for the determination of enzymatic activity, together with a description of the units employed, are described in detail in reference 1.

are given in Table I. It is seen that removal of the polymorphonuclear cells by Seitz filtration caused the disappearance of tryptic activity.

In order to ensure that the removal of the tryptic activity by Seitz filtration was not due to adsorption of enzyme by the filter pad, we investigated the effect of centrifugation on the tryptic activity of fluids from patients with purulent meningitis. In addition, the sediment resulting from the centrifugation of one fluid was taken up with an amount of physiologic saline such that the volume of the resulting suspension was equal to that of the original fluid sample. The antitryptic power of the various portions was also determined. The results are given in Table II.

TABLE II

PATIENT	PORTION STUDIED	DIAGNOSIS	DESCRIPTION OF FLUID	CELL COUNT (POLYS.)	TRYPSIN (UNITS)	ANTI-TRYPSIN UNITS %
1. A. McQ.	(a) Original fluid	Influenzal meningitis	Lumbar, turbid	7,200	0.65	40
	(b) After centrifugation		Clear		0.0	40
2. A. McQ.	(a) Original fluid	Influenzal meningitis	Cisternal, opalescent	2,000	1.20	8
	(b) After centrifugation		Clear		0.03	8
3. R. W.	(a) Original fluid	Influenzal meningitis	Lumbar, turbid	5,000	1.40	34
	(b) After centrifugation		Clear		0.25	40
	(c) Sediment taken up with NaCl		Turbid		1.20	0

In each case the tryptic activity of the fluid was greatly decreased by centrifugation, while the antitryptic power was unaffected. In the case of R. W. nearly all the tryptic activity was found in the sediment.

Since the results indicated clearly that the source of the trypsin of the cerebrospinal fluid of purulent meningitis is the polymorphonuclear pleocytosis, we thought that extension of the centrifugation studies to other enzymes might yield information about their sources. It was difficult to obtain sufficient fluid at a single puncture for such studies in addition to the routine cellular, chemical, and bacteriologic studies. However, we succeeded in obtaining enough data to indicate approximately the relative importance of the different sources of the enzyme studied. In Table III are given the data for 5 fluids.

The results for trypsin agree with those of the previous experiments and require no further discussion at this point. In 4 out of 5 fluids centrifugation caused a marked decrease in the phosphatase activity, while in one fluid the activities of the original fluid, supernatant fluid after centrifugation, and sediment were practically equal. The reductions in the phosphatase activity of the fluids were relatively less than the corresponding reductions in tryptic activity. Lipolytic activity was reduced by centrifugation. The activity of the supernatant fluid varied from 0 to about 50 per cent of that of the original fluid, while the average sediment activity was about 50 per cent that of the original

fluid. Tributyrinase activity in the fluid was reduced by centrifugation to values from 50 to 93 per cent of those of the original fluid. The sediment had about half as much activity as the original fluid. Results obtained with esterase and amylase were similar to those obtained with tributyrinase, except for some instances where relatively low enzyme values were not affected by centrifugation. The antitryptic power of the fluids studied was not decreased by centrifugation. In fact, in one case the fluid after centrifugation had a very high antitryptic effect (82 units), and a much lower effect (14 units) when cells were present. In no case was any antitrypsin found in the sediment. If there is any antitryptic substance in the cells, its effect is entirely overcome by the polymorphonuclear trypsin.

The effect of centrifugation on the enzymatic activity of the cerebrospinal fluid of purulent meningitis can perhaps be most easily understood in terms of Willstätter's work on the lyo and desmo forms of leucocytic trypsin and amylase. Willstätter<sup>11</sup> has distinguished between two types of leucocytic enzymes according to the ease with which they are extracted from the cellular material. Lyoenzymes, which are easily extracted from the cells, are considered to be freely dissolved in the cells. Desmoenzymes, which are extracted with greater difficulty, are thought to be firmly bound to the cellular protoplasm. The desmoenzymes are almost always bound to cell complexes and are active in this condition. According to Willstätter and Rohdewald,<sup>12</sup> only about 7 to 11 per cent of the trypsin of polymorphonuclear leucocytes occurs as lyotrypsin, the remainder occurs in the desmo form. This would explain why removal of the polymorphonuclear cells by centrifugation removes nearly all of the tryptic activity of the fluid. At most, the fluid after centrifugation had 15 to 17 per cent of the tryptic activity of the original fluid; the average tryptic activity after centrifugation was about 9 per cent of the original activity, agreeing very well with the lyotrypsin values given by Willstätter and Rohdewald.

The work of Willstätter and Rohdewald<sup>11</sup> on leucocytic amylase indicated that this enzyme occurs to a much greater extent in the lyo form than does leucocytic trypsin. Our results with the amylase of meningitis cerebrospinal fluid are in agreement with this finding, since the sediment after centrifugation had relatively less, and the clear liquor more, activity than was the case with trypsin.

At this point an interesting application of our work can be made. The fact that centrifugation of fluid removes a large part of the phosphatase activity indicates that phosphatase is firmly bound in the polymorphonuclear leucocyte, probably more so than amylase, but less firmly than trypsin. Also, our results indicate that the polymorphonuclear cells are very rich in lipase, tributyrinase, and esterase, in agreement with the findings of Fleischmann.<sup>13</sup> Further, the fact that the sediment studied after centrifugation contains these enzymes in considerable quantity indicates that they occur to an important extent in the desmo form. It is difficult, however, to obtain any idea of the relative amounts of lyo and desmo forms of leucocytic lipase, tributyrinase, and esterase, since we have thus far been unable to distinguish between these lyoenzymes and

plasma enzymes which may have entered the cerebrospinal fluid because of increased meningeal permeability.

*B. Effect of Increased Meningeal Permeability: Antitrypsin.*—It is well known that there is an increase in meningeal permeability in meningitis.<sup>14</sup> It is possible, therefore, that some enzymes may enter the cerebrospinal fluid from the blood plasma in meningitis. So far, no direct measure of the enzymatic activity derived from this source is available. However, study of the antitrypsin (or trypsin-inhibitor) of meningitic fluid may throw some light on this subject.

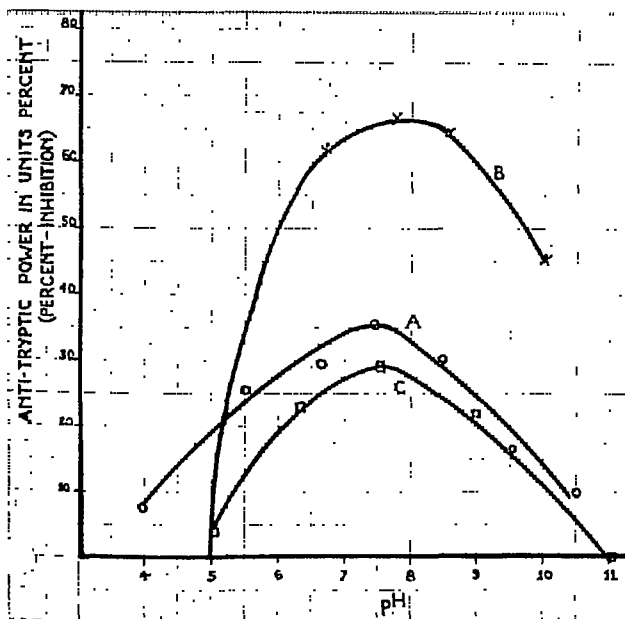


Fig. 1.

Chrometzka<sup>15</sup> has found that the antitryptic power of blood serum varies with pH, having a maximum value of approximately pH 8. On either side of this region the antitryptic power decreases until it disappears at pH values 4 and 12. It is interesting to see whether the antitrypsin found in meningitic cerebrospinal fluid depends in a similar way on pH. If so, it would point to the hematogenous origin of the pathologic antitrypsin, particularly since no antitrypsin is found in normal cerebrospinal fluid.

Accordingly, we studied the effect of change of pH on the antitryptic power of fluid from a patient with hydrocephalus complicated by *Staphylococcus*

TABLE III

CASE	DIAGNOSIS	PORTION STUDIED	APPEARANCE	CELL COUNT	TRYPSIN (MM TYRO- SINE)	ANTITRYP- SIN UNITS	PHOS- PHATASE (MG. PHOS- PHORS)	LIPASE (MM FATTY ACID)	TRIG- LYC- LASE (MM FATTY ACID)	ESTER- ASE (MM MALTOSE)	AMYLASE (MM MALTOSE)
D. R. (1)	Meningococci meningitis	(a) Lumbar fluid (b) Supernatant fluid aft- er centrifugation	Turbid Clear	Poly. 3,000	0.67 0.63	7 10	14.4 5.2	2.5 1.3	2.2 1.4	0.6 0.9	0 0
D. R. (2)	Meningococci meningitis	(a) Lumbar fluid (b) Supernatant fluid aft- er centrifugation	Cloudy Clear	Poly. 1,200	0.40 0	0 0	8.0 1.3	1.3 0.7	3.4 1.7	0.9 0.9	0.8 0.8
S. K. (1)	Hydrocephalus B. pyocyaneus pyocephalus	(a) Ventricular fluid (b) Supernatant fluid aft- er centrifugation (c) Sediment taken up with NaCl	Turbid Bluish green Turbid	Frank pus	2.80 0.43 2.33	14 82 0	33.6 9.6 60.2	1.3 0 0.3	9.8 9.1 4.7	4.2 2.9 2.5	19.1 8.8 9.9
R. K. (2)	Hydrocephalus B. pyocyaneus pyocephalus	(a) Ventricular fluid (b) Supernatant fluid aft- er centrifugation (c) Sediment taken up with NaCl	Turbid Bluish green Turbid	Frank pus	2.32 0.27	26 0	66.4 22.9	1.1 0.2	7.4 4.9	3.9 1.6	0
W. B.	Cerebral abscess	(a) Lumbar fluid (b) Supernatant fluid aft- er centrifugation (c) Sediment taken up with NaCl	Turbid Clear Turbid	Poly. 34,930			55.0 55.4 52.4	2.4 0.4 1.3	5.0 3.3 3.7	3.9 1.7 3.5	

*aureus pyocephalus*. The fluid was divided into several portions to which were added 1 or 2 drops of sodium hydroxide or hydrochloric acid solutions of different strength. After ten or fifteen minutes the antitryptic power of the various portions was determined by the method described in our previous paper. The results are plotted as curve A in Fig. 1. It is seen that the antitryptic power has a maximum of approximately pH 7.5 and falls off on either side of this estimate. At pII values of 4 and 11 the antitryptic power is small but has not completely disappeared. It was thought that this may have been due to the short time (ten to fifteen minutes) allowed for the action of the acid and alkali on the cerebrospinal fluid. The work was repeated on two other fluids, one from the same patient, and the other from a patient with a clinical diagnosis of tuberculous meningitis. In these instances the fluid portions were treated with acid or alkali for an hour. The results are plotted as curves B and C of Fig. 1. It can be seen that the antitryptic power has a maximum value after incubation at pII 7.0 to 8.0 and falls off on either side of this range, vanishing at about pH 4 and pH 11.

It follows from the foregoing results that the antitryptic power of meningitic cerebrospinal fluid depends on pII in the same way as the blood serum antitrypsin. Since the normal cerebrospinal fluid contains no antitryptic substances, the antitrypsin of meningitic fluid must come either from the blood plasma or from the polymorphonuclear leucocytes. However, it has been found (see Tables II and III) that centrifugation of meningitic fluid has no effect on the antitryptic power of the fluid, and that the cellular sediment itself exhibited no such power. It is probable, therefore, that the antitrypsin of meningitic cerebrospinal fluid enters the fluid from the plasma because of the increased meningeal permeability.

C. *Enzymes of the Cerebrospinal Fluid in Tuberculous Meningitis*.—Since the cerebrospinal fluid of tuberculous meningitis differs from that of purulent meningitis in the number of polymorphonuclear cells, it would be expected that this difference might account for the observed difference in enzymatic properties. This is borne out by the fact that the polymorphonuclear cells have been shown to be responsible for the presence of tryptic activity and to a large extent for the presence of phosphatase. The enzymatic activity of the cerebrospinal fluid of tuberculous meningitis, which is not due to the polymorphonuclear cells, is probably due in part to the passage of plasma enzymes across the meningeal barrier, and in part to the presence of lymphocytes which are known to be particularly rich in lipolytic enzymes.<sup>16</sup>

### III. ENZYMES OF THE CEREBROSPINAL FLUID IN HYDROCEPHALUS AND BRAIN TUMOR

We have pointed out<sup>1</sup> that our specimens of cerebrospinal fluid in hydrocephalus, brain tumor, brain abscess, and brain cyst can be divided into two groups. The first group contains those cases in which meninges or ventricles are known to have been invaded by a neoplasm, or by hemorrhage, or inflamed. The second group contains cases in which there was no known involvement of meninges or ventricles. Some typical examples of fluids of the two groups are given in Table IV.

TABLE IV

CASE	DIAGNOSIS	APPEARANCE	CELL COUNT	TRYPSIN (MM TYROSINE)	ANTI- TRYPSIN UNITS	PHOS- PHATASE (MG. PHOS- PHORUS)	LIPASE TRIBUTYRINASE ESTERASE (MM FATTY ACID)			AMYLASE (MM MALTOSE)	TOTAL PROTEIN MG. PER CENT
							0.5	2.6	0	0.7	613
W. W.	Hydrocephalus medullo- blastoma of cerebel- lum, invasion of fourth ventricle	Xanthochromic, lumbar	Lymph. 40	0	<i>First Subgroup</i>						
					93	5.8					
B. K.	Hydrocephalus, B. pyo- cynaeus pyocephalus, spina bifida	Frank pus, ventricular		2.32	26	66.4	1.1	7.4	3.9	0	2,500
W. H.	Hydrocephalus, Staph. aureus pyocephalus, spina bifida	Opalescent, ventricular	Poly. 310	0.04	65	6.9	1.0	2.1	0	0	680
J. M.	Hydrocephalus, glioma of cerebellum, no invasion of ventricles or menin- ges	Clear, ventricu- lar	Lymph. 3	0	<i>Second Subgroup</i>		5.4	2.3	0	0	13
					0	0.8					
(1) M. R.	Hydrocephalus	Clear lumbar	Lymph. 20	0	0	1.0	0.8	3.0	0		16
(2) M. R.	Hydrocephalus	Clear lumbar	Lymph. 1	0	6	1.1	0.4	1.3	0	0	17
B. S.	Hydrocephalus	Clear ventricu- lar		0	0	0.6	3.0	1.4	0	0	41

As the data indicate, fluids of the first group are usually abnormal in appearance, have elevated cellular and protein contents, and have high phosphatase, lipase, tributyrinase, and antitrypsin values. The fluids of the second group are normal, except for high lipase and tributyrinase values. The enzymatic properties of fluids of the first group can, to some extent, be explained by the presence of polymorphonuclear leucocytes, or by invasion of the meninges by a tumor. The lipase and tributyrinase of fluids of the second group cannot be accounted for on these bases since there is no significant pleocytosis and no increased meningeal permeability (i.e., antitrypsin, phosphatase values, total protein are normal). It is necessary, therefore, to seek a different source for these enzymes. In the following discussion we shall suggest the possibility that these enzymes may be associated with degeneration of nervous tissue.

The pathology of the progressive cerebral atrophy secondary to hydrocephalus has been described by Penfield,<sup>17</sup> by Penfield and Elvidge,<sup>18</sup> and by Hassin.<sup>19</sup> The lateral ventricles are greatly distended. There is much nerve tissue destruction, that of the white matter next to the ventricles being more rapid than that of the gray matter. The corpus callosum is raised and thinned, while the septum pellucidum often disappears. In severe hydrocephalus the corpus callosum may be entirely obliterated, the subcortical white matter destroyed, and the optic chiasm, optic tracts, and tuber cinereum extremely flattened.<sup>20-22</sup> Large numbers of gitter cells are found in the corpus callosum, cerebral parenchyma, subependymal areas, and Virchow-Robin spaces. Hassin<sup>22</sup> has studied the histologic changes in the brain secondary to the increased intracranial pressure caused by extracerebral as well as intracerebral tumors. He found diffuse degenerative changes in the corpus callosum, optic nerve, chiasm, and optic tract, that is, the changes were similar to those found in the pressure atrophy of hydrocephalus.

Penfield<sup>23</sup> has studied the formation and activity of gitter cells in areas of cerebral softening surrounding a glioma. These cells contain neutral fats, fatty acids, and other products of the (enzymatic) breakdown of nerve tissue and demyelination. They carry their contents to the adventitial spaces of the blood vessels where they lose their granular and vacuolar appearance, decrease in size, and finally leave the vicinity of the vessels in characteristic form for renewed phagocytosis. Apparently some of the digestive contents of the gitter cells pass into the perivascular spaces from which they are transported in the tissue fluids to the subarachnoid space. It is possible that in this way lyolipolytic enzymes of the gitter cells and myelophages may reach the cerebrospinal fluid.

Another possible explanation of the presence of lipolytic enzymes is in terms of the enzymes of the brain tissue itself. Recent unpublished experiments in our laboratory indicate that fresh minced brain tissue possesses considerable lipolytic activity. It seems possible that the destructive processes may cause the liberation of lipolytic enzymes from the tissue. The enzyme may then reach the cerebrospinal fluid as indicated above. In this connection it is interesting to note that Spiegel-Adolf<sup>24</sup> has recently reported that in certain neurologic conditions apparently normal fluids may show evidences of brain tissue destruction when conductivity and interferometric methods of analysis are used. Some



cases of hydrocephalus and brain tumor in her series have protein values which are within normal limits, but, nevertheless, have abnormal "residual interferometric values," indicating brain tissue destruction. These findings seem to be in agreement with those reported in our study.

If the lipolytic activity of the fluids under discussion is due to brain tissue destruction, it would be reasonable to expect increased lipolytic activity of the cerebrospinal fluid in other degenerative diseases of the cerebrospinal fluid, such as multiple sclerosis, Schilder's disease, and subacute combined degeneration of the cord, which are characterized by extravascular demyelination and glial proliferation.

More complete studies of the lipolytic activity, neutral fat, fatty acid, cholesterol, and cholesterol ester contents of cerebrospinal fluid in these diseases may yield valuable information about the chemistry and pathology of nerve tissue degeneration. The fact that high lipase values have been found in isolated cases of multiple sclerosis, carbon monoxide poisoning, and cerebral arteriosclerosis indicates that such studies may be profitable.

The study of multiple sclerosis by such an approach may be of particular interest in view of Brickner's suggestion<sup>24</sup> that the demyelination in this disease is caused by a lipolytic ferment. It is possible that the lipase and tributyrinase which we have found in the cerebrospinal fluid and which we suggest may be involved in nerve tissue degeneration may be related to Brickner's lipolytic enzyme.

#### CONCLUSIONS

1. There are two possible sources of the enzymes of normal cerebrospinal fluid: (a) the blood plasma and (b) the neural tissue fluid. It is at present impossible to determine the relative importance of the two sources.

2. Centrifugation studies of the cerebrospinal fluid of purulent meningitis indicate: (a) that the tryptic activity of the fluid is due almost entirely to the desmotrypsin of the polymorphonuclear cells; (b) that the phosphatase activity of the fluid is due chiefly to desmophosphatase of the polymorphonuclear cells; (c) that the lipase, tributyrinase, and esterase activities are due in part (about 50 per cent) to desmolipolytic enzymes of the polymorphonuclear cells; (d) that the amylase activity is due, in a lesser degree, to the desmoamylases of the polymorphonuclear cells.

3. The remainder of the enzymatic activity of the cerebrospinal fluid of purulent meningitis is probably due to: (a) lyoenzymes of the polymorphonuclear cells and (b) plasma enzymes which enter the cerebrospinal fluid because of increased meningeal permeability. It has so far been impossible to determine the relative importance of (a) and (b).

4. The antitryptic power of the cerebrospinal fluid of tuberculous meningitis and pyocephalus varies with pH, having a maximum near pH 7.5 to 8.0, and decreasing on either side of that range. This similarity to the behavior of serum antitrypsin suggests that the cerebrospinal fluid antitrypsin enters the cerebrospinal fluid from the plasma because of increased meningeal permeability.

5. The lipase and tributyrinase of the cerebrospinal fluid of hydrocephalus and brain tumor in cases where there is no known involvement of the meninges

or ventricles may in part be lyoenzymes of the gutter cells and myelophages which take part in the phagocytosis of fatty debris of nerve tissue degeneration. Lipolytic enzymes may also be liberated from brain tissue by destructive processes. The enzymes possibly reach the cerebrospinal fluid via the perivascular spaces.

6. In cases of hydrocephalus and brain tumor where the meninges are involved by tumor or inflammation, cells or increased meningeal permeability may cause an increase in the enzymatic activity of the cerebrospinal fluid.

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# ON THE VALUE OF A "REPEAT" INJECTION OF TETANUS TOXOID (SECONDARY STIMULUS) IN ACTIVE IMMUNIZATION AGAINST TETANUS

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IN A PREVIOUS communication<sup>1</sup> we reported the results obtained after active immunization of 34 adults by means of a primary course of two injections of tetanus toxoid, alum-precipitated, refined. We now wish to report our follow-up observations on 20 members of this group who were subjected to a third "repeat" (secondary stimulus) injection of alum toxoid. Their immunologic response to the primary course of immunization is shown in Table I.

Before the third dose of 1.0 c.c. of tetanus toxoid, alum-precipitated, refined, was injected, all patients were bled, and the sera were titrated for tetanus antitoxin. All but two showed less than 0.1 unit per cubic centimeter. Cases 9925 and 9953 had 0.1 unit each. Sixteen subjects received the secondary stimulus 272 days after the second dose of toxoid. Four got the third injection 359 days after the primary course of immunization was completed.

A composite picture of antitoxin production was then obtained through serial titrations (Table II).

Of 8 patients tested on the fourth day after the "repeat" injection, only one showed 0.25 unit. The rest had the same control values of less than 0.10 unit. Of 9 subjects tested on the fifth day, all but one showed 0.1 unit or more per cubic centimeter of blood serum. The exception, Case 9941, showed less than 0.1 unit. However, when retested on the seventh day, this patient had 1.0 unit of antitoxin per cubic centimeter of serum. It is interesting to note the definite increase in antitoxin titer that occurred within 48 hours in Cases 9928, 9932 and 10004. On the sixth day all subjects but one showed good protection; Case 9947, who showed less than 0.10 unit, was not retested again until a month had elapsed when he had 0.25 unit. This subject was the only member of the group who showed a rather poor antitoxin response, since he dropped to less than 0.1 unit when retested 183 days after the third injection of toxoid. On the seventh day 15 subjects were tested. They all showed a marked increase in their antitoxin titer, the average value being well above 2.0 units per cubic centimeter of blood serum. The magnitude of the increase in many cases was at least 50 times the control value. Between the sixth or seventh and the thirtieth day following injection of the secondary stimulus, 11 subjects showed a further increase in titer, 4 had the same antitoxin level, and 1 person showed a decrease in value. Four individuals actually tested 5 units or more per cubic centimeter of blood serum. To obtain this amount of antitoxin in the blood by passive immunization, a hypodermic injection of about 75,000 units of tetanus antitoxin is needed.

TABLE I

TETANUS TOXOID, ALUM-PRECIPITATED, REFINED No. 5969-1

(Two Doses Given 92 Days Apart)

Titer expressed in units of tetanus antitoxin per c.c. of blood serum

CASE	CONTROL TITER	DAYS AFTER FIRST DOSE TITER	DAYS AFTER SECOND DOSE TITER				
			7 days	15 days	91 days	181 days	272 days
9945†	+0.01	30 days 0.10	-0.10	+0.25	0.01	0.003	0.01
9939†	0.003	30 days -0.003	15 days +0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9942†	+0.003 -0.01	53 days -0.01	15 days +0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
10002*	0.003	92 days -0.01	15 days 0.10	91 days 0.01	181 days 0.003	272 days +0.01 -0.10	
9928†	+0.003	53 days 0.01	7 days +0.25	91 days +0.25 -0.50	181 days 0.10	272 days +0.01 -0.10	359 days +0.01 -0.10
9932†	+0.003 -0.01	30 days 0.003	7 days +0.25	153 days 0.10	181 days 0.10	272 days +0.01 -0.10	359 days +0.01 -0.10
10004*	0.003	30 days 0.003	15 days +1.0 -5.0	91 days 0.50	181 days +0.10 -0.25	272 days +0.01 -0.10	359 days +0.01 -0.10
9931†	0.003	30 days 0.01	15 days +0.25	91 days +0.25 -0.50	181 days 0.10	272 days +0.01 -0.10	359 days +0.01 -0.10
9943†	0.003	68 days -0.003	15 days +0.25	91 days 0.10	181 days +0.01 -0.10	272 days +0.01 -0.10	
9946†	+0.01	68 days -0.01	15 days +0.25	91 days +0.01 -0.10	181 days +0.01 -0.10	272 days +0.01 -0.10	
9948*	-0.003	30 days -0.003	7 days 0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9933†	0.003	30 days 0.003	7 days +0.25	91 days 0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9941*	0.003	30 days -0.003	7 days +0.25	91 days 0.10	181 days +0.01 -0.10	272 days +0.01 -0.10	
9944*	+0.003	53 days -0.01	7 days -0.10	15 days +0.25	91 days +0.003 -0.01	181 days +0.01 -0.10	272 days 0.01
9935*	+0.003 -0.01	30 days -0.003	15 days +0.10 -0.25	122 days +0.01 -0.10	153 days 0.01	181 days 0.01	272 days 0.01
9955†	-0.003	68 days -0.01	15 days 0.25	91 days +0.25 -0.50	181 days +0.01 -0.10	272 days +0.01 -0.10	
9947*	+0.01	92 days 0.01	15 days +0.10 -0.25	91 days 0.01	181 days 0.003	272 days 0.01	
9954*	0.003	53 days 0.01	15 days 2.0	122 days +0.10 -0.25	181 days +0.01 -0.10	272 days +0.01 -0.10	
9925†	+0.003 -0.01	30 days +0.01 -0.10	7 days 0.10	91 days 0.25	181 days +0.01 -0.10	272 days 0.10	
9953*	0.003	68 days 0.01	15 days +1.0 -5.0	91 days 0.25	181 days +0.01 -0.10	272 days 0.10	

\*Each dose consisted of 0.5 c.c. of alum toxoid.

†Each dose consisted of 1.0 c.c. of alum toxoid.

TABLE II  
TETANUS TOXOID, ALUM-PRECIPITATED, REFINED No. 8387-3  
(Third "Repeat" Dose of 1.0 c.c. Given 272 Days After Basic Course of Immunization Was Completed)  
Titer expressed in units of tetanus antitoxin per c.c. of blood serum

CASE	TITER BEFORE THIRD DOSE	DAYS AFTER THIRD OR "REPEAT" DOSE														TITER
		TITER														
		4 DAYS	5 DAYS	6 DAYS	7 DAYS	30 DAYS	45 DAYS	92 DAYS	183 DAYS	274 DAYS	367 DAYS	464 DAYS	560 DAYS			
9945†	0.01	-	-	+0.25 -0.50	+1.0 -3.0	-	0.50	+0.10 -0.25	0.10	0.10	+0.01 -0.10	-				
9939†	+0.01 -0.10	-	-	+2.0 -3.5	+1.0 -2.0	-	+0.50 -1.0	+0.10 -0.25	+0.10 -0.25	+0.10 -0.25	+0.10 -0.25	0.10	+0.25 -0.50	0.10		
9942†	+0.01 -0.10	-	-	+1.0 -2.0	-	+2.0 -3.5	2.0	+0.50 -1.0	0.50	0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50
10002*	+0.01 -0.10	-	-	+0.25 -0.50	+0.25 -0.50	-	+0.10 -0.25	0.10	0.10	0.10	+0.01 -0.10	+0.01 -0.10	-			
9928†	+0.01 -0.10	0.10	-	5.0	+5.0 -8.0	-	3.0	1.5	+1.5 -3.0	+0.50 -1.0	-	-	+0.50 -1.0	-		
9932†	+0.01 -0.10	1.0	-	+3.0 -5.0	+3.0 -5.0	-	+0.50 -1.0	0.50	0.50	0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50
10004*†	+0.01 -0.10	+0.10 -0.25	-	+1.0 -3.0	5.0	-	+1.0 -3.0	0.50	+0.10 -0.25	+0.10 -0.25	+0.50	+0.50	+0.50	+0.50	+0.50	+0.50

\*Basic course of immunization.

\*Basic course of immunization: 2 doses, 0.5 c.c. each, given 92 days apart.  
†Basic course of immunization: 2 doses, 1.0 c.c. each, given 92 days apart.  
††Third dose ("repeat") given 350 days after basic course of immunization was completed.  
Alum toxoid No. 5069-1 used.

9931†	+0.01 -0.10	0.25	1.0	-	+3.0 -5.0	-	+1.0 -3.0	1.0	+0.50 -1.0	0.50	-	+0.25 -0.50
9943†	+0.01 -0.10	-	+0.25 -0.50	-	+2.0 -5.0	-	+0.50 -1.0	+0.25 -0.50	+0.25 -0.50	0.25	0.25	+0.10 -0.25
9946†	+0.01 -0.10	-	0.25	-	+1.0 -2.0	-	+0.25 -0.50	0.25	+0.10 -0.25	0.10	+0.01 -0.10	-
9948*	+0.01 -0.10	-	+0.25 -0.50	-	+3.0 -5.0	-	+1.0 -3.0	+0.50 1.0	0.50	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50
9933†	+0.01 -0.10	-	+0.25 -0.50	-	3.0	-	+1.0 -3.0	+0.25 -0.50	0.50	+0.25 -0.50	+0.25 -0.50	+0.10 -0.25
9941*	+0.01 -0.10	-	+0.01 -0.10	-	1.0	-	1.0	0.50	0.25	+0.10 -0.25	+0.10 -0.25	+0.10 -0.25
9944*	0.01	-	-	+0.25 -0.50	-	-	0.25	0.10	0.10	+0.01 -0.10	+0.01 -0.10	-
9935*	0.01	-	-	+0.10 -0.25	-	-	-	-	-	-	-	-
9955†	+0.01 -0.10	-	-	0.25	-	-	+0.50 -1.0	+0.25 -0.50	0.25	+0.10 -0.25	+0.10 -0.25	0.10
9947*	0.01	-	-	-0.10	-	-	0.25	+0.01 -0.10	+0.01 -0.10	+0.01 -0.10	+0.01 -0.10	-
9954*	+0.01 -0.10	-	-	-	+1.0 -2.0	3.0	+1.0 -3.0	+1.0 -2.0	2.0	+0.50 -1.0	+0.50 -1.0	+1.0
9925†	0.10	-	-	+1.0 -3.0	-	-	+1.0 -2.0	+0.25 -0.50	1.0	+0.25 -0.50	+0.25 -0.50	+0.25 -0.50
9953*	0.10	-	-	-	+1.0 -3.0	2.0	1.0	0.50	0.25	0.25	+0.10 -0.25	0.25

When retested 90 days after the "repeat" injection, there was noted a definite decrease in the antitoxin titer in practically all cases, although it was still well above the minimum protective level of 0.1 unit. Thereafter a more gradual loss of antitoxin occurred. At the end of one year only 3 out of 19 subjects showed less than 0.1 unit of antitoxin per cubic centimeter. Three months later two additional subjects dropped to less than 0.1 unit. The latest titration, done 18.5 months after injection of the third dose of alum toxoid, showed that 14 members of this group still retained a protective titer of 0.1 unit or more, with an average of more than 0.25 unit per cubic centimeter.

#### COMMENT

The injection of a third dose of alum toxoid in subjects who had been previously actively immunized against tetanus is associated with a rise in antitoxin titer of a surprisingly high magnitude. This rise takes about five days to reach the level of 0.1 unit which is present in the blood serum of persons passively immunized with 1,500 units of tetanus antitoxin.<sup>2</sup> It is stated that the period of incubation of tetanus in man is usually from six to fourteen days, and is directly proportional to the amount of toxin and the severity of the disease. However, shorter periods of incubation, six days or less, are known to occur. It is reasonable to expect that the injection of the "repeat" dose or secondary stimulus will prevent these cases of acute tetanus (short incubation), since we are dealing here with a rapidly increasing titer due to active immunity. Also the value of 0.1 unit which we believe necessary for protection against tetanus<sup>3</sup> is rather a conservative one.

A comparison of Tables I and II reveals the interesting fact that in this series of cases the injection of the "repeat" dose was followed by an antitoxin immunity that persisted above the 0.1 unit level for a definitely longer period of time than was the case after the primary course of immunization. Thus, after the latter (Table I) 5 subjects out of 17 dropped to less than 0.1 unit when tested three months after the second dose, 16 subjects out of 20 showed less than 0.1 unit when bled six months after the second injection of toxoid, while 18 members of the group had less than 0.1 unit when tested nine months after completion of the course. On the other hand, after the "repeat" injection of toxoid was administered to this group of patients, only one subject out of 19 dropped to less than 0.1 unit when tested six months later. Previous titrations showed that all subjects enjoyed good protection. This prolonged maintenance of a protective titer is also reflected in subsequent tests. Thus 14 subjects still showed 0.1 unit or more per cubic centimeter of blood serum as late as eighteen months after the injection of the secondary stimulus.

Although the number of subjects studied is small, this finding of a more prolonged antitoxin response following injection of the secondary stimulus is highly significant, and may have important practical implications if confirmed in a large series of cases. In our original study<sup>1</sup> we found that subjects who received 2 doses, 1.0 c.c. each, of alum toxoid seemed to develop a higher and more lasting antitoxin titer than those who received 2 doses of 0.5 c.c. each. It is of interest to note that following the third "repeat" injection of alum

toxoid, the 3 subjects who dropped to less than 0.1 unit before the year was up, were persons who had received 2 doses of 0.5 c.c. for their primary course of injections. In the absence of a skin test to determine the state of antitoxin immunity, the individual variability in antitoxin response makes it necessary to administer 1.0 c.c. of tetanus toxoid, alum-precipitated, whenever an injury occurs after completion of the primary course of immunization. This naturally brings up the question of sensitization. The toxoid does not contain any horse serum, but it does have traces of beef broth and peptone which may give rise to allergic reactions in sensitive subjects. Fortunately, the number of such cases is small. It must be remembered, however, that alum preparations of protein materials have been successfully used in the laboratory to induce anaphylactic shock in experimental animals. Whether this can occur in man is not known. In our experience covering immunization of over 200 people in the last five years, no untoward reaction has been encountered, except an outbreak of urticaria in one patient following the second injection of toxoid.

#### CONCLUSIONS

1. One cubic centimeter of tetanus toxoid, alum-precipitated, refined, can be safely injected as a secondary stimulus in subjects who had previously undergone primary immunization against tetanus antitoxin by means of two injections of alum toxoid.

2. Five to seven days elapsed before the antitoxin level of the blood serum reached the minimum protective value of 0.1 unit, following injection of this secondary stimulus ("repeat" or third dose).

3. The injection of this secondary stimulus produced in seven to thirty days a very marked increase in the antitoxin content of the blood. The magnitude of this increase was in many cases at least 50 times the control value. Several subjects had 5.0 units or more of tetanus antitoxin per cubic centimeter of blood serum.

4. Following injection of this secondary stimulus, the antitoxin titer appeared to be maintained above the minimum protective level of 0.1 unit for a longer period of time than was the case after the primary course of two toxoid injections.

5. The occurrence of an injury in subjects that have undergone primary immunization against tetanus requires the injection of a secondary or "repeat" stimulus (1.0 c.c. of alum toxoid).

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# THE HISTIDINE CONTENT ("DIAZO VALUE") OF THE BLOOD IN PEPTIC ULCER\*

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THE recent introduction of histidine (Larostidine) therapy for peptic ulcer has been followed by both favorable<sup>1</sup> and unfavorable<sup>2</sup> reports in the literature. A satisfactory explanation of the mechanism or mode of action of injected histidine in experimental or human peptic ulcer is still missing. While the amino acid deficiency theory of Weiss and Aron<sup>3</sup> is attractive, direct experimental proof of its correctness is not available as yet. Chemical experimentation along this line has been meager and inconclusive, due, in part, to the absence of a specific and completely satisfactory method for determining the histidine content of the blood.<sup>4-7</sup>

Diazonium salts have been frequently used to determine colorimetrically certain substances, such as phenols, imidazoles, tyrosine, etc., which, as a group, constitute the "diazio value" of the blood.<sup>8</sup> In a recent paper from this laboratory it was shown that ether-soluble phenols constitute but an insignificant portion of this "diazio value."<sup>9</sup> Schwartz, Riegert, and Bricka<sup>10</sup> state that normal human blood contains no phenols and that histidine is the most abundant imidazole. It seems apparent that any appreciable fluctuations in the histidine content of the blood should be reflected in corresponding changes in the "diazio value."

## METHOD

With this idea in mind, the experimental methods outlined in the previous paper<sup>9</sup> were applied to a series of fasting bloods obtained from patients with peptic ulcer who had been admitted to the hospital for treatment. Twenty-four patients, most of whom had duodenal ulcer, were subjected to study. Each of these patients had complained of "stomach trouble," indigestion, epigastric distress, hyperacidity, and other typical ulcer symptoms for periods extending from one to fifteen years. Their ages ranged from 22 to 58 years. The clinical diagnosis in each case was confirmed by roentgen examination. The chemical analyses were generally made shortly after admittance to the hospital. With each ulcer blood, a pooled specimen of oxalated, human blood secured from the hospital laboratory was subjected to simultaneous analysis. The filtrates were prepared by laking 1 volume of blood with 2 volumes of water, followed by 1 volume of 10 per cent sodium tungstate and then by 1 volume of  $\frac{2}{3}$  N sulfuric acid. It is important to add the reagents in this order, since it was observed that the "diazio values" tend to run about 10 per cent higher if the acid is added to the laked blood before the sodium tungstate. Presumably added free amino and other reactive groups are opened up by the strong acid. It was also found that the precipitated blood proteins are removable either by

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TABLE I  
HISTIDINE CONTENT ("DIAZO VALUE") OF ULCER AND CONTROL BLOODS

NO.	AGE	SEX	PERIOD OF ULCER SYM- TOMS (YEARS)	NONPROTEIN NITROGEN MG./100 C.C.				AMINO ACID NITROGEN MG./100 C.C.				BLOOD "DIAZO VALUES"				DIAGNOSIS		
				ULCER BLOOD		CON- TROL BLOOD		ULCER BLOOD		CON- TROL BLOOD		KOESSLER- HANKS METHOD AS HISTIDINE MG./100 C.C.		P-NITROANILINE METHOD AS HISTIDINE MG./100 C.C.			MESCHKOWA METHOD AS HISTIDINE MG./100 C.C.	
				ULCER BLOOD	CON- TROL BLOOD	ULCER BLOOD	CON- TROL BLOOD	ULCER BLOOD	CON- TROL BLOOD	ULCER BLOOD	CON- TROL BLOOD	ULCER BLOOD	CON- TROL BLOOD	ULCER BLOOD	CON- TROL BLOOD			
1	25	M	3	39.5	41.7	7.0	7.0	1.2	1.2	4.8	5.5	3.0	2.2	3.0	2.2	Bleeding gastric ulcer		
2	58	M	15	50.0	33.0	6.1	6.1	1.9	1.6	5.6	4.5	2.4	2.2	2.4	2.2	Gastric ulcer with hemorrhage		
3		M		22.0				1.5		4.3						Gastric ulcer		
4	56	M	3	38.7	36.4	7.2	7.2	1.4	1.4	6.0	6.4	3.0	2.8	3.0	2.8	Gastric ulcer		
5	26	M	2	37.5	39.1	6.4	6.3	1.1	1.1	4.7	4.9	3.0	2.2	3.0	2.2	Duodenal ulcer		
6	24	F	1	31.6	36.4	6.4	7.2	1.4	1.4	5.7	6.4	2.8	2.9	2.8	2.9	Ovarian cyst, possible duodenal ulcer		
7	38	M	2	30.3	36.0	6.9	7.0	1.3	1.4	6.0	6.3	2.4	2.7	2.4	2.7	Duodenal ulcer with hemorrhage		
8	48	M	2	31.3		6.3		1.0		4.3		2.3		2.3		Duodenal ulcer with hemorrhage		
9	22	M	4	35.3	33.0	7.0	7.1	1.5	1.4	6.2	6.4	2.8	2.9	2.8	2.9	Duodenal ulcer		
10	37	F	1	33.3	35.3	6.7	6.9	1.4	1.4	6.2	6.4	2.6	2.8	2.6	2.8	Bleeding duodenal ulcer		
11	27	M	5			7.4	7.8	2.1	1.7	6.2	5.1	3.4	2.6	3.4	2.6	Duodenal ulcer		
12	33	M	10					1.6	1.7	4.5	6.0		2.3		2.3	Pyloric ulcer with partial ob- struction		
13	31	M	7	38.3	41.7	6.8	5.5	1.8	1.9	4.3	4.7	2.1	2.2	2.1	2.2	Duodenal ulcer, nonobstructive		
14	36	M	4	30.0		5.4	5.9	1.6	1.6	4.5	4.8	2.0	2.3	2.0	2.3	Chronic spastic colon; gastroin- testinal neurosis		
15	27	M	5	31.0	28.0	6.4	6.0	1.9	1.6	5.4	4.5	3.0	2.2	3.0	2.2	Duodenal ulcer		
16	38	F	3	34.0	34.1	6.5	5.8	1.2	1.2	4.5	4.7	2.1	2.4	2.1	2.4	Duodenal ulcer		
17	33	M	2	37.5	30.0	7.3	6.8	1.7	1.4	6.0	5.1	3.2	2.4	3.2	2.4	Duodenal ulcer		
18	45	M	2	20.4	31.3	7.0	6.9	1.5	1.4	4.7	4.9	2.2	2.4	2.2	2.4	Duodenal ulcer		
19	50	M	5	30.0	30.0	6.7	6.8	1.5	1.4	5.5	4.9	2.4	2.3	2.4	2.3	Duodenal ulcer		
20	58	M	2	37.5	30.2	5.6	5.5	1.5	1.3	5.7	4.9	2.5	2.5	2.5	2.5	Nonobstructive duodenal ulcer		
21	28	M	2	33.6	30.0	6.5	5.5	1.3	1.3	5.4	4.9	2.4	2.5	2.4	2.5	Peptic ulcer following posterior gastroenterostomy		
22																Peptic ulcer		
23	40	M	5	31.3	32.6	5.1	5.6	1.3	1.5	4.7	4.9	2.2	2.5	2.2	2.5	Old cicatrized duodenal ulcer		
				33.6	35.0	6.3	7.2	1.1	1.2	4.0	4.5	2.3	2.5	2.3	2.5	with pyloric obstruction		
24	22	M	1	33.3		6.3	5.2	1.0	1.1	5.0	4.5	2.4	2.4	2.4	2.4	Nonobstructive duodenal ulcer		
Averages				34.05	34.11	6.56	6.44	1.45	1.42	5.18	5.24	2.57	2.46					

filtration or by centrifugation without influencing the various values. In addition, determinations of the nonprotein nitrogen and amino acid nitrogen content of the bloods were also made by the usual Folin colorimetric methods. The data are given in Table I.

#### COMMENT AND SUMMARY

It is obvious that the values given by the various chemical determinations on the pathologic and on mixed control bloods tend to run parallel. Significant differences between the bloods from ulcer patients and the control bloods are consistently absent, and all values are well within normal limits. The amino acid nitrogen values for the ulcer bloods ranged from 5.1 to 7.5, with an average of 6.56, whereas those for the control bloods ranged from 5.2 to 7.8, with an average of 6.44 mg. per 100 c.c.<sup>4, 5, 12</sup> The "diazo values," as determined by the modified Koessler-Hanke method,<sup>9</sup> and calculated and reported as histidine, ranged from 1.0 to 2.1, with an average of 1.45 mg., and 1.1 to 1.9, with an average of 1.42 mg. histidine per 100 c.c. for the ulcer and control bloods, respectively. By the p-nitroaniline method,<sup>11</sup> values for the ulcer bloods ranged from 4.0 to 6.2, with an average of 5.18 mg. histidine, as compared with 4.5 to 6.4, with an average of 5.24 mg. histidine per 100 c.c. for the control bloods. By the modified Meschkowa procedure<sup>9</sup> ulcer bloods ranged from 2.0 to 3.4, with an average of 2.57 mg., as compared with 2.2 to 2.9, with an average of 2.46 mg. histidine per 100 c.c. for the control bloods. These values are similar to those reported for tungstic acid filtrates in the previous study on the phenol and imidazole content of normal human blood.<sup>9</sup>

Examination of the data shows that no distinguishing differences exist between the values given by the various diazotization procedures on the control bloods and on those from patients with peptic ulcer. There seems to be no appreciable difference between the histidine content of the blood of patients with peptic ulcer and the blood of normal individuals. The data do not tend to confirm the theory that a histidine deficiency exists in the blood of patients with peptic ulcer.

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# LABORATORY METHODS

## A CONTRIBUTION TO THE QUESTION OF THE DETERMINATION OF NICOTINIC ACID IN URINE

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THE papers of Elvehjem, Fouts, Smith, Spies and his collaborators directed attention to the therapeutic significance of nicotinic acid. It has been said that this substance contains the most effective protective factor against pellagra known. It is no longer doubtful that deficiency of this substance may give rise to pictures which represent the individual symptoms of pellagra, and in the dog this deficiency is able to bring about the black tongue disease. The most simple method of recording these stages clinically would be to determine the content of nicotinic acid of the body fluids.

It was, therefore, natural that attempts were made to obtain more exact data on the occurrence of nicotinic acid in the normal and pathologic human organism. The first investigations on the occurrence of nicotinic acid in the urine are connected with the names of Ackermann, Komori, and Sendju. These workers observed that nicotinic acid, after having been fed to the dog, was excreted partly as free nicotinic acid, partly combined with glycine (as nicotinuric acid) and as trigonelline. As to the methods, the papers of Karrer and Keller opened new paths describing a quantitative method of determining nicotinic acid amide in the organism of the animal by means of dinitrochlorobenzene. At the same time, Vilter, Spies, and Mathews reported that they, too, had succeeded in determining nicotinic acid and some of its derivatives in the urine by means of dinitrochlorobenzene. After having carried out exact experiments these authors found that nicotinic acid, nicotine amide, sodium nicotinate, and nicotinic acid diethylamide produce a substance with dinitrochlorobenzene which yields a red color on the addition of alkali. Neither trigonelline nor picolinic acid give this red color reaction. While Karrer and Keller remarked that they obtained deep-colored yellowish-red derivatives after alkalization, Spies and his co-workers stated that they observed, after addition of alkali, a purple-burgundy red color, in proportion to the quantity of derivatives of nicotinic acid present. If the added alkali is too much concentrated, the color changes to yellow. Moreover, Spies and his co-workers pointed out that the red color tints of nicotinic acid and nicotinic amide differ from each other, even if the two substances have undergone the same preliminary treatment. This occurrence made it necessary to have two different standard color curves for quantitative colorimetric measurement. With reference to the urine examination, this fact represents an obstacle,

since the presence of different shades of red renders an unmistakable result doubtful. It has not yet been discussed as to what the different red color obtained with dinitrochlorobenzene may be due. All determinations carried out hitherto have had the result that the normal human urine contains 20 to 50 mg. of nicotinic acid in the daily amount.

We also tried to attain a method of determination of nicotinic acid in the urine with dinitrochlorobenzene. Short of slight modifications we proceeded along the same lines as described by Spies. After publication of his method, however, we strictly followed his instructions.

The analysis of the normal individual components of the urine likely to produce the red color after addition of dinitrochlorobenzene, led us, after a number of negative results had been obtained, to a positive one. We found that creatinine yielded a red color with dinitrochlorobenzene in proportion to the concentration of creatinine. But also with creatinine the red color does not appear until alkali has been added to the mixture. The more the creatinine solution is diluted, the more the shade changes from purple to orange. With the help of this method it is possible to determine the presence of amounts of creatinine up to 0.5 mg. (We used creatinine hydrochloride produced by Hoffmann La-Roche, Basle.)

We believe that the fact that creatinine yields a color reaction with dinitrochlorobenzene provides a source of error with reference to the determination of nicotinic acid in the urine, which is performed by means of this reagent. Considering that 500 to 1,000 mg. of creatinine are excreted in the urine daily, while only 20 to 50 mg. of nicotinic acid are discharged, and the presence of both substances can be proved to the same extent, creatinine is, therefore, present in amounts 20 to 50 times as great as the other substance. In order to obtain definite results for nicotinic acid itself, creatinine must be removed from the urine. Even by using Lloyd's reagent we have so far not succeeded in finding a reliable method.

ALFASIROAD, P.O.B. 1139

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*Addendum.*—After this report was completed, E. Bandier and J. Hald (*Biochem. J.* 33: 264, 1939) and K. Ritsert (*Klin. Wchnschr.* 18: 934, 1939) published papers which provided important information on the problem of determining nicotinic acid in biological material (including blood and urine). Bandier and his collaborators devised a method in which they used cyanogen-bromide-metol (p-methylaminophenol sulfate) while Ritsert suggested a modification of the brom-cyananiline method. Ritsert's results greatly differ from those of other authors on quantitative lines. For example, he found in the normal urine an average proportion of 50-300 gamma in 100 c.c. which would amount to 1-6 mg. in the daily output.

## AN IMPROVED SUCTION APPARATUS\*

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**W**ATER-SUCTION siphonage has been a recognized therapeutic procedure in surgery for many years. Wangenstein<sup>1</sup> used it with a Levin tube for gastrointestinal decompression. It has also been employed for biliary drainage, suprapubic drainage, stomach drainage, and for many other purposes. Meehling<sup>2</sup> in 1917 described the earliest suction apparatus using water siphonage. In some of the suction apparatus the drainage mixed with the water system. This made an unsightly appearance and did not permit accurate measurement of the amount of siphonage returns. This was corrected by creating a separate water system and a flask for siphonage collection. There are numerous suction siphonage machines that have been patented. These are expensive and involve the use of valves that make its use complicated.

At the University Hospital we have devised an apparatus that is very simple in construction, easily transportable, and inexpensive. It does not require much attention while in operation. The necessary parts are as follows:

1. Two 2,000 c.c. flasks, graduated and capable of being suspended, inverted.
2. Three 2-holed rubber stoppers.
3. Glass tubing, rubber tubing, and glass connecting tips.
4. One liter flask to collect siphonage returns.
5. Metal standard, 54 inches high.
6. One Y glass tube.
7. Metal stopcocks (screw type) and right angle clamps to suspend flasks.

The two 2,000 c.c. flasks comprise the water system which is entirely separate from the siphonage returns. The apparatus is constructed as shown in Fig. 1.

The first 2,000 c.c. flask (*A*) is filled with water to a level just below the breather tube and suspended from the top of the standard. The second 2,000 c.c. flask (*B*) is empty and suspended at the bottom of the standard. The 54 inch standard allows adequate height for efficient suction. The liter flask (*C*) is supported in the middle of the standard. Each 2,000 c.c. flask has a 2-holed rubber stopper, in one hole of which there is a long glass breather tube and in the other a shorter glass connecting tip. On the collecting liter flask there is a 2-holed rubber stopper; in one hole there is a glass Y tube, and in the other a small glass connecting tip. The system is so arranged that water will flow from flask *A* at the top of the standard into flask *B* at the bottom of the

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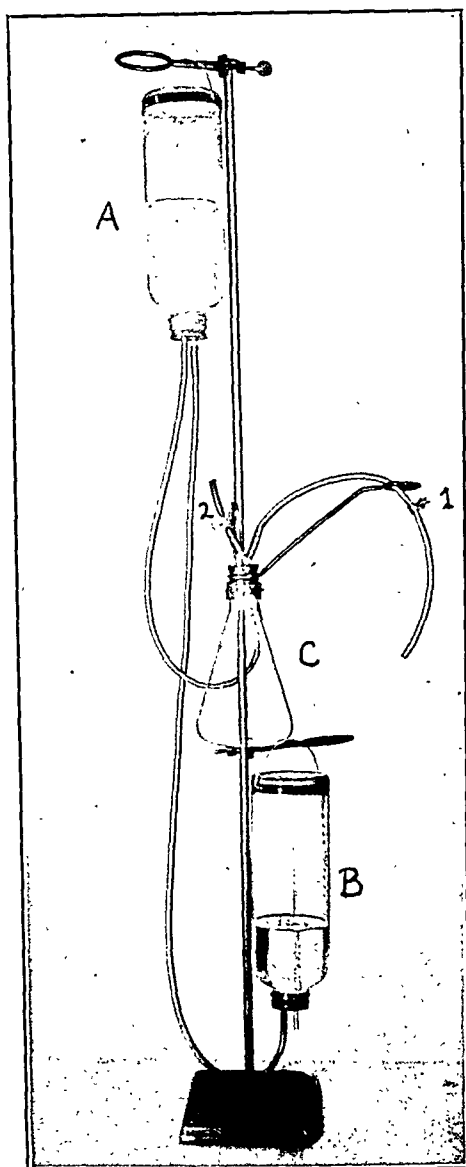


Fig. 1.

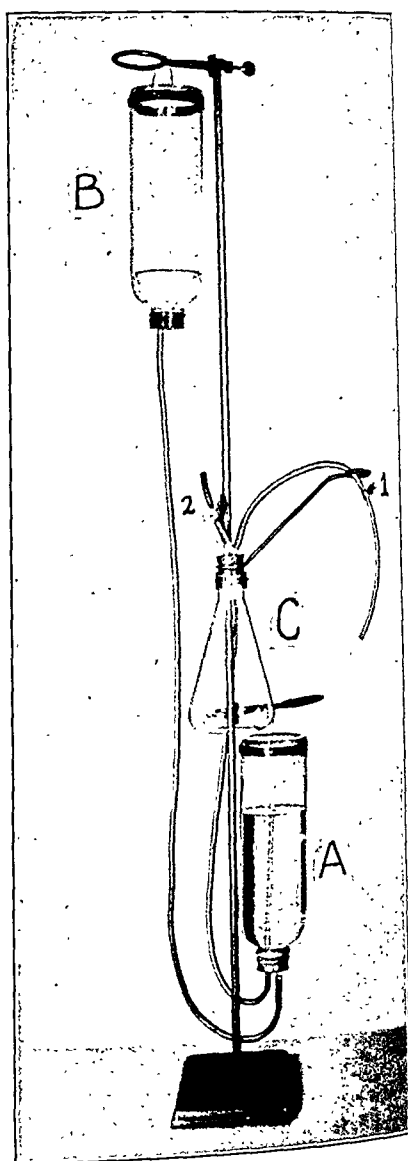


Fig. 2.

Fig. 1.—Apparatus is ready for connection to Levin tube. Flask A is at the top of standard, flask B is at bottom, and flask C is in the middle. Stopcock 2 is on one limb of the Y tube, and stopcock 1 is on tube connecting with Levin tube. Stopcock 2 is closed and stopcock 1 is open. Water is running from flask A into flask B and creating suction in flask C, which is connected to the Levin tube and stomach.

Fig. 2.—Refill the apparatus when flask A is empty, then hang flask A at bottom of standard and raise flask B to the top of standard. Close stopcock 1 and open stopcock 2. It takes one minute to refill flask A without disconnecting the entire apparatus or adding any more water into the system. When flask A is refilled, then raise it to top of standard and lower flask B to the bottom; close stopcock 2 and open 1. The apparatus is again ready for use.

standard. As the water flows from flask *A* a vacuum is created above the breather tube in flask *A* that is transmitted to flask *C* which is connected with the Levin tube and stomach. There are two stopcocks in the entire system, one on the tube connecting with the Levin tube (1), and the other on one limb of the Y tube. When in operation, stopcock 1 is open and stopcock 2 on Y tube is closed. To refill flask *A*, when the water in flask *A* has emptied into flask *B* one opens stopcock 2 and hangs flask *A* at the bottom of the standard and flask *B* at the top. It takes about one minute to refill the flask without disconnecting any stoppers or adding any more water into the system. When flask *A* is full again, resuspend at the top of the standard, close stopcock 2, and open stopcock 1. The system is ready to operate again.

The system will operate for twelve hours and longer without requiring refilling, as each flask holds 2,000 c.c. In refilling it is important to see that stopcock 2 is kept open and stopcock 1 is closed. This prevents air being pumped into the stomach during the water change.

The advantages of this apparatus are as follows:

1. It can be constructed readily and inexpensively by anyone.
2. It does not require much attention from the nurse or person in attendance.
3. The apparatus is complete on one standard and light enough to permit ease in transportation.
4. Accurate measurement of fluid and air siphonage can be made.
5. The water system does not require removal of stoppers and refilling of the flasks.
6. The standard is set on the floor and the drainage returns are out of the patient's sight.

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# AN IMPROVED METHOD FOR THE IODIMETRIC DETERMINATION OF PYRUVIC ACID\*

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**B**ECAUSE of the important position which pyruvic acid occupies in metabolism, a reliable method for its chemical determination should be available. Acidimetric analyses of biologic mixtures being necessarily impractical, it appears that a method based upon the reactivity of the carbonyl group should offer a better approach. Unfortunately, there is no known compound which is specific in its reaction with the carbonyl group of pyruvic acid. Consequently, if other alpha keto acids, ketones, or aldehydes are present, they will be included in the analysis. Despite this lack of specificity, a measurement of the total carbonyl content of a biologic mixture affords a valuable means of following the course of pyruvate metabolism. If a more positive identification of the carbonyl content is required, it is necessary to resort to characterization studies.

The most widely used procedure for the quantitative determination of pyruvic acid is based on the iodimetric estimation of the bisulfite taken up by the carbonyl group. Such has been the basis of the Clift and Cook method.<sup>1</sup> Tests of this method, using pure pyruvic acid, gave uncertain results because of a fading end point when the excess bisulfite was removed with iodine. Moreover, only about 83 per cent of the expected carbonyl content could be determined, as compared with carboxyl determinations giving 98 per cent. The fading end point suggested that the bisulfite-pyruvate solution was unstable at room temperature (24.5° to 33.0° C.), which was in agreement with the observation of Elliott and co-workers.<sup>2</sup> Cooling the bicarbonate, 0.1 N iodine, and the bisulfite-pyruvate solutions to ice temperature<sup>2</sup> completely stabilized the end point and increased the carbonyl value by about 8 per cent. The carbonyl value, however, was still appreciably lower than the carboxyl value.

It seemed that the effervescence occurring when bicarbonate was added to free the bound bisulfite might cause a loss of some of the liberated bisulfite, even in the cold solution. To test this possibility, an excess of 0.01 N iodine was added to the cold bisulfite-pyruvate solution after the initial end point had been reached but prior to liberation of the bound bisulfite with bicarbonate; the excess iodine was titrated against standard thiosulfate. This change in the procedure raised the carbonyl value to about 99 per cent and thus brought it into good agreement with the carboxyl value. The original method<sup>1</sup> was further improved by a material decrease in the amount of bisulfite, with a consequent decrease in total volume and in the amount of 0.1 N iodine required to combine with the excess bisulfite.

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The method as finally adopted, together with tests of its reliability and its application to certain biologic materials, is given below.

#### DESCRIPTION OF METHOD

*Reagents.*—(A) 2.5 per cent sodium bisulfite, prepared daily or at frequent intervals.

(B) 0.01 N potassium iodate. 0.3567 gm. of potassium iodate was dissolved in water and diluted to one liter. This served as the primary standard. The solution is stable for several months, if kept in the dark.

(C) 0.1 N iodine (approx.). 3.57 gm. potassium iodate, 45 gm. potassium iodide, and 5 ml. concentrated sulfuric acid per liter of solution.

(D) 0.01 N iodine, prepared daily or at frequent intervals from (C) by dilution.

(E) 0.1 N sodium thiosulfate (approx.). 25.0 gm.  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$  per liter of solution, containing 0.5 per cent amyl alcohol.<sup>3</sup>

(F) 0.01 N sodium thiosulfate, prepared daily or at frequent intervals from (E) by dilution.

(G) 2.5 per cent potassium iodide.

(H) 5.0 per cent acetic acid. 50 ml. glacial acetic acid diluted to one liter.

(I) Starch solution. A 1.0 per cent solution of soluble starch in nearly saturated sodium chloride. Solution of the starch was accomplished by heating to near boiling.

(J) Sodium bicarbonate suspension. An excess of sodium bicarbonate was added to water.

(K) Purified pyruvic acid. Eastman C. P. pyruvic acid was purified by triple vacuum distillation in an all-glass still. The third distillation which had a boiling point of 41.5 to 42.5° C. (not corrected) was collected in a flask immersed in an ice bath. A 10.0 per cent water solution was prepared as a stock solution. It was stored in a dark bottle at 3° C., the bottle being covered with a heavy cloth.

The water used in all analytical work was distilled water which was further purified by redistilling over slightly alkaline potassium permanganate.

*Procedure.*—*Standardization of 0.01 N Sodium Thiosulfate.*—A 10.0 ml. aliquot of 0.01 N potassium iodate (B) was measured (Ostwald-Folin pipette) into a 6 inch by 1 inch test tube. Five milliliters of 2.5 per cent potassium iodide (G) and 3 ml. of 5 per cent acetic acid (H) were added, and it was titrated immediately with sodium thiosulfate from a 10 ml. burette until the solution became a faint yellow color. Two drops of starch solution (I) were now added, and the titration continued to a faint-blue end point. The titrations were made in triplicate.

*Standardization of 0.01 N Iodine.*—This was standardized by titration with the 0.01 N sodium thiosulfate. Triplicate or quadruplicate portions, measured from a 10 ml. burette, were used. The titration was carried to the same faint-blue end point as for the sodium thiosulfate standardization.

TABLE I

THE EFFECT OF COOLING THE REAGENTS AND OF ADDING AN EXCESS OF 0.01 N IODINE UPON THE CARBOXYL VALUE OF A PURE PYRUVIC ACID SOLUTION\*

CONDITIONS OF TEST	TIME AFTER SOLUTION WAS PREPARED	NUMBER OF ALIQUOTS	PYRUVIC ACID		
			AMOUNT IN ALIQUOT†	AMOUNT RECOVERED	
	<i>Days</i>		<i>Mg.</i>	<i>Mg.</i>	<i>Per Cent</i>
Reagents at room temperature	0	6	2.50	2.07	82.8
	7	6	2.50	2.12	84.8
	14	6	2.50	2.01	80.4
	21	6	2.50	2.06	82.4
	28	6	2.50	2.10	84.0
Reagents at ice temperature	0	6	2.50	2.34	93.6
	7	6	2.50	2.27	90.8
	14	6	2.50	2.19	87.6
	21	6	2.50	2.17	86.8
	28	6	2.50	2.35	94.0
Reagents at ice temperature; excess 0.01 N iodine	0	6	2.50	2.49	99.6
	7	6	2.50	2.50	100.0
	14	6	2.50	2.49	99.6
	21	6	2.50	2.47	98.8
	22	6	2.50	2.48	99.2
	28	6	2.50	2.46	98.4
	86	6	2.50	2.45	98.0
	105	6	2.50	2.37	94.8

\*Pyruvic acid made to 0.05 N with hydrochloric acid, prior to carboxyl determination.

†Carboxyl titrations were made on triplicate 0.5 gm. samples of pyruvic acid, using 0.5 N sodium hydroxide with methyl red as the indicator. The carboxyl values at 0, 7, 14, 21, and 28 days were 97.9, 98.4, 98.4, 98.4, and 98.3 per cent, respectively.

TABLE II

EFFECT OF ACIDITY UPON PYRUVATE DETERMINATION\*

EFFECT OF ACIDITY UPON PYRUVIC ACID					
ACIDITY†	NUMBER OF ALIQUOTS	PYRUVIC ACID			COMMENT
		AMOUNT IN ALIQUOT	AMOUNT RECOVERED		
<i>Norm.</i>		<i>Mg.</i>	<i>Mg.</i>	<i>Per Cent</i>	
0.0057‡	4	2.50	2.45	98.0	No effervescence
0.01	4	2.50	2.51	100.4	No effervescence
0.025	4	2.50	2.48	99.2	Very slight effervescence
0.05	4	2.50	2.47	98.8	Slight effervescence
0.10	4	2.50	2.50	100.0	Some effervescence
0.50	4	2.50	2.50	100.0	Appreciable effervescence; less 0.1 N iodine required
1.00	4	2.50	2.40	96.0	Very appreciable effervescence; much less 0.1 N iodine required
					temperature

\*Excess 0.01 N iodine added before sodium bicarbonate addition. Ice temperature reagents.

†Hydrochloric acid was used.

‡No hydrochloric acid was added.

TABLE III

EFFECT OF THE VOLUME OF SODIUM BISULFITE-PYRUVATE SOLUTION UPON PYRUVATE RECOVERY\*

TOTAL VOLUME OF SOLUTION	NUMBER OF ALIQUOTS	PYRUVIC ACID		
		AMOUNT IN ALIQUOT	AMOUNT RECOVERED	
<i>Ml.</i>		<i>Mg.</i>	<i>Mg.</i>	<i>Per Cent</i>
2.0	5	2.50	2.53	101.2
4.0	5	2.50	2.52	100.8
6.0	5	2.50	2.55	102.0
11.0	5	2.50	2.50	100.0
20.0	5	2.50	2.50	100.0

\*An acidity of 0.05 N with hydrochloric acid; excess 0.01 N iodine added before sodium bicarbonate addition; ice temperature reagents.

*Carbonyl Determination.*—1. To each of as many 6 inch by 1 inch test tubes as required, placed in an ice bath, was added 1 ml. of 2.5 per cent sodium bisulfite (A). This is sufficient bisulfite to react with about 20 mg. of pyruvic acid.

2. Five milliliter aliquots of pyruvic acid or unknown, containing from 0.1 to 5.0 mg. of pyruvic acid, were added (Ostwald-Folin pipette). All samples containing an unknown amount of pyruvic acid were analyzed in quadruplicate.

3. The solutions were mixed thoroughly and allowed to stand in an ice bath for fifteen minutes or more.

4. Two drops of starch indicator (I) were then added.

5. Ice temperature 0.1 N iodine was added from a pipette until the excess sodium bisulfite was dissipated; a slight excess of iodine, equivalent to about 1 ml. of 0.01 N sodium thiosulfate, was added. A maximum of 4.3 ml. of 0.1 N iodine will be required. Steps 4 and 5 may be carried out on several samples at one time, so long as the analyses are completed within an hour.

6. By means of the 0.01 N iodine and 0.01 N sodium thiosulfate solutions, contained in 10 ml. burettes, the end point was adjusted to any selected shade of faint-blue color.

7. An amount of 0.01 N iodine equivalent to the pyruvate-bound bisulfite, together with an excess of about 1 ml., was added. If the sample contained an unknown amount of pyruvic acid, one aliquot was analyzed by the Elliott and "associates"<sup>2</sup> modification of the Clift and Cook procedure<sup>1</sup> so as to ascertain the approximate volume of 0.01 N iodine required by each of the remaining three aliquots.

8. The ice temperature sodium bicarbonate suspension (J) was thoroughly mixed and 5 ml. was added.

9. The excess 0.01 N iodine was titrated with the 0.01 N sodium thiosulfate, and the end point was adjusted to the same shade of blue color as in step 6. This end point should be permanent for at least thirty seconds.

10. The amount of pyruvic acid was calculated by multiplying the net milliliters of exactly 0.01 N iodine by 0.44.

*Blanks on Reagents.*—These were made according to the same procedure as for the carbonyl determinations, except that 5.0 ml. of 0.05 N hydrochloric acid replaced the pyruvic acid or unknown in step 2. The average value for 30 determinations was equivalent to 0.098 ml. of 0.01 N iodine (Table V).

#### TESTS OF THE METHOD

The effect of cooling the reagents and of adding an excess of 0.01 N iodine prior to liberation of the bound bisulfite with bicarbonate is clearly shown by the data in Table I. The analyses were made on the purified acid (K). In addition, the data indicate a very slow deterioration in the pyruvic acid; the carbonyl content had decreased by about 5 per cent at the end of 105 days. Biologic tests also showed a somewhat greater toxicity with decrease in carbonyl content.

Clift and Cook<sup>1</sup> suggested that the reaction between bisulfite and various aldehydes and ketones was more nearly quantitative at relatively low acidities.

Their results indicated that the acidity for pyruvic acid determination should not be greater than 0.1 N. The data in Table II show that the addition of excess 0.01 N iodine prior to bisulfite liberation and the use of cold reagents permitted the quantitative recovery of a constant amount of pyruvic acid over a considerable range of acidity. Even though the acidity was great enough to cause a loss of free bisulfite at 0.5 N acidity, the bound bisulfite was not affected. However, when the solution was adjusted to 1.0 N, there was a slight loss in bound bisulfite as well as an appreciable loss in free bisulfite. The wide range in acidity, 0.01 to 0.5 N, is of value in biologic determinations because it eliminates the necessity for accurately adjusting the acidity of the solutions to be analyzed.

Although a final volume of 6.0 ml. is recommended in the procedure, the data in Table III show that this exact volume is not required, if the amount of pyruvic acid remains constant. No difference was found by varying the volume of the bisulfite-pyruvate solution from 2.0 to 20.0 ml. However, with the use of the suggested apparatus and concentration of reagents, as well as for a possibly better uniformity in results, it would be well to use not over 10.0 ml. aliquots of the unknown.

The data given in the various tables were largely obtained without allowing the solution containing an excess of 0.1 N iodine to stand after its addition. The main purpose for such a suggested addition to the procedure is for convenience only; it is time-saving to add the starch indicator and 0.1 N iodine to several samples at one time and then complete the titration. The data in Table IV show that thirty minutes' standing with excess iodine did not greatly

TABLE IV

THE EFFECT OF EXCESS IODINE UPON THE STABILITY OF BISULFITE-PYRUVATE SOLUTION

MATERIAL	EXCESS 0.1 N IODINE IN TERMS OF 0.01 N Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	TIME OF STANDING WITH EXCESS IODINE	NUMBER OF ALIQUOTS	PYRUVIC ACID		
				AMOUNT IN ALiquot	AMOUNT RECOVERED	
	ML.	Hr.		Mg.	Mg.	Per Cent
Pure pyruvic acid*†	0.10-1.50	0-0	6	2.50	2.48	99.2
	1.15-1.50	0.4-0.8	6	2.50	2.49	99.6
	0.75-1.50	2.0-3.0	6	2.50	2.40	96.0
Rat urines†	1.0-2.0	0-0	7	-	0.72	-
	1.0-2.0	0.4-0.8	7	-	0.71	-
	1.0-2.0	2.0-3.0	7	-	0.67	-
Rat urines†	0.1-1.7	0-0	6	-	1.63	-
	1.3-2.0	0.4-0.8	6	-	1.66	-
	1.9-3.0	2.0-3.0	6	-	1.38	-

\*Acidity of 0.05 N with hydrochloric acid.

†Ice-temperature reagents.

affect the results, but that two to three hours' standing may lower the apparent pyruvic acid content. This result indicates that the bisulfite-pyruvate solution may slowly react with the iodine. The same results were obtained from a mixture of rat urines as for pure pyruvic acid.

As a final test of the method, pyruvic acid in amounts of from 0.05 to 15.0 mg. was determined in quadruplicate. Two series of analyses were made; in

one series the regular procedure was followed, whereas in the other series an excess of 0.1 N iodine was added and allowed to stand for about thirty minutes. Inasmuch as there was no essential difference in the results obtained from the two series, the results were averaged and are presented in Table V. Definitely low results were obtained with the smallest (0.05 mg.) and largest (15.0 mg.) amounts of pyruvic acid. Although 97.1 per cent of the pyruvic acid was recovered in the 10.0 mg. level, yet 0.3 mg. was lost, whereas in the 0.05 mg. level only 0.01 mg. was lost. However, with the concentration of reagents and apparatus used, the method appeared to be more accurate for amounts of from 0.1 mg. to 5.0 mg. of pyruvic acid. If smaller or larger quantities are to be determined, the concentration of the reagents and the apparatus should be changed so that quantitative recoveries would be obtained.

TABLE V  
THE DETERMINATION OF VARIOUS QUANTITIES OF PYRUVIC ACID\*

NUMBER OF ALIQUOTS	NET VOLUME 0.01 N IODINE	PYRUVIC ACID		
		AMOUNT IN ALIQUOT	AMOUNT RECOVERED	
	Ml.	Mg.	Mg.	Per Cent
30	0.098	0.00	-	-
8	0.092	0.05	0.04	80.0
8	0.223	0.10	0.10	100.0
8	0.563	0.25	0.25	100.0
8	1.135	0.50	0.50	100.0
8	2.290	1.0	1.01	101.0
8	3.307	1.5	1.46	97.3
8	5.584	2.5	2.46	98.4
8	11.298	5.0	4.97	99.4
8	22.069	10.0	9.71	97.1
4	31.957	15.0	14.06	93.7

\*Average of two series of determinations. An acidity of 0.05 N with hydrochloric acid and 5.0 ml. aliquots of pyruvic acid; ice-temperature reagents; excess 0.01 N iodine.

#### APPLICATION TO CERTAIN BIOLOGIC MATERIALS

The method has been used in this laboratory in a study of pyruvate metabolism in the intact rat following pyruvate treatment.<sup>4</sup> This work was conducted as individual balance tests covering an eight-hour metabolism period, during which time the animals received 6 doses of sodium pyruvate. The amount of pyruvic acid apparently not retained by the animal was determined by analysis of the urine and of the contents of the intestinal tract.

The urine was collected over a small amount (about 0.5 gm.) of trichloroacetic acid. The rat was then killed and the intestinal tract with contents was removed, finely minced, washed into a small flask, and about 1 gm. of trichloroacetic acid was added. The samples were allowed to stand overnight in a refrigerator and were then filtered individually into 100 ml. volumetric flasks. After thorough washing, the samples were adjusted to volume. For the determination of the pyruvic acid (total bisulfite-binding substances) content, 5.0 ml. aliquots were used; this volume, therefore, represented one-twentieth of the sample. No adjustment of the acidity of the sample was made.

The data in Table VI give the amounts of apparent pyruvic acid (total bisulfite-binding substances) unabsorbed and excreted by normal young rats

TABLE VI

EFFECT OF EXCESS 0.01 N IODINE UPON THE APPARENT PYRUVIC ACID CONTENT OF RAT URINE AND INTESTINAL CONTENT FOLLOWING PYRUVATE TREATMENT\*

BIOLOGIC MATERIAL	SERIES†	NUMBER OF RATS	WEIGHT OF RATS	PYRUVIC ACID GIVEN	APPARENT PYRUVIC ACID PER RAT‡	
					NO EXCESS 0.01 N IODINE	EXCESS 0.01 N IODINE
Urine	A	10	Gm. 55.4	Mg. 0.0	Mg. 1.17	Mg. 1.63
	B	17	60.0	413.6	20.46	23.22
	C	4	55.6	585.0	58.33	61.43
	D	14	65.9	865.7	98.18	104.46
Intestinal content	A	10	55.4	0.0	1.32	1.96
	B	17	60.0	413.6	1.80	2.81
	C	4	55.6	585.0	7.79	8.69
	D	14	65.9	865.7	53.45	58.61

\*Ice-temperature reagents.

†The pyruvic acid was given as the nearly neutralized sodium salt at levels of 0, 60, 90, and 120 mg. in 3.0 ml. per 50 gm. of rat for series A, B, C, and D, respectively. The metabolism period covered 8 hours, during which time each rat received 6 treatments. The rats were killed 1.5 hours after the final treatment.

‡Total bisulfite-binding substances, calculated as pyruvic acid.

following several levels of pyruvic acid treatment. Although it is uncertain that the bisulfite-binding substances found in the urine and intestinal content of the control rats (series A) was pyruvic acid, the increases following pyruvic acid treatment can reasonably be attributed to the administered pyruvate. As with the purified pyruvic acid (Table I), the addition of an excess of 0.01 N iodine prior to liberation of the bound bisulfite increased the amount of apparent pyruvic acid. Furthermore, in similar levels the increase was of the same order of magnitude as for the pure acid.

#### SUMMARY

1. An improved iodimetric procedure for pyruvic acid determinations in solutions of pure pyruvic acid or in certain biologic materials is described.

2. The essential improvements are (a) the addition of an excess of 0.01 N iodine prior to liberation of pyruvate-bound bisulfite with bicarbonate; (b) a decrease in the amount of bisulfite with a consequent decrease in amount of 0.1 N iodine and in total volume of solution; (c) the use of 0.01 N sodium thiosulfate which permitted a more accurate adjustment of the end point prior to and following the liberation of pyruvate-bound bisulfite.

3. With the apparatus used and concentration of reagents employed, 0.10 to 5.0 mg. of pyruvic acid can be quite accurately determined.

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## PROTHROMBIN TIME DETERMINATION\*

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THE increasing interest in vitamin K (the coagulation vitamin) warrants the publication at this time of the procedures used in the recent studies on the prothrombin time determination and on vitamin K therapy, as reported by Waddell, Bray, Guerry, and Kelley.<sup>1-3</sup> The method of determining the prothrombin time is essentially that described by Quick,<sup>4</sup> with certain modifications designed to make it more acceptable as a routine procedure in the average clinical laboratory. Especially useful is the modification suggested by one of us (Bray<sup>3</sup>) using capillary blood in the study of prothrombin levels in the newly born infant.

### PREPARATION OF THROMBOPLASTIN

The brain is removed from a rabbit recently killed by etherizing, or by bleeding, or by injecting air into the veins; brains of rabbits killed by a blow on the head are not satisfactory. The meninges and the blood vessels are carefully removed. The brain is then macerated by means of a spatula and spread in a thin layer over the surface of a glass plate. The macerated brain is dried in the incubator at 37° C. for two to three days.

The dried brain is removed from the glass by scraping with a razor blade and then finely chopped with the blade. This gives a fine flaky material, which is more satisfactory than the compact powder obtained when the dried brain is ground in a mortar. This material keeps well in the dry state at room temperature. Some batches have kept their potency for over two months. The acetone method described by Quick<sup>4</sup> also has been found satisfactory.

*Titration of the Thromboplastin.*—A 1:20 suspension of thromboplastin is made in physiologic salt solution (0.9 per cent), 0.2 gm. of the dried brain in 3.8 c.c. of the salt solution is ample for the titration. The suspension is heated at 56° C. for ten minutes, with occasional stirring. The material is then centrifugalized at low speed for a few minutes to throw down the coarse particles. A turbid supernatant fluid is obtained, containing fine particles in suspension. The following dilutions of thromboplastin are set up in 0.5 c.c. amounts: 1:20, 1:30, 1:40, 1:60, 1:80, 1:120, and 1:160. This may be conveniently done in two stages:

(1) Four tubes are set up in a rack, and into the first and second are placed 0.5 c.c. of the 1:20 dilution (i.e., 0.5 c.c. of the supernatant fluid). Into

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tubes 2, 3, and 4 are placed 0.5 c.c. of 0.9 per cent salt solution. From tube 2 after mixing, 0.5 c.c. is transferred to tube 3, and so on, 0.5 c.c. being discarded from the last tube. These dilutions are 1:20, 1:40, 1:80, and 1:160.

(2) Three other tubes are set up. Into each tube is placed 0.5 c.c. of 0.9 per cent salt solution. Into tube 1 is placed 1.0 c.c. of the 1:20 dilution, and after mixing, 0.5 c.c. is discarded and 0.5 c.c. is transferred to tube 2; after mixing, 0.5 c.c. is transferred to tube 3; and after mixing, 0.5 c.c. is discarded. These dilutions are 1:30, 1:60, and 1:120. The dilutions are then arranged in ascending order.

Two or more samples of blood with known prothrombin time, or from normal healthy adults are used for the titration. The titrations are run in duplicate as outlined under "Technique of Prothrombin Time Determination." The titration usually shows an optimum zone of low findings. For routine tests we choose the dilution falling in the middle of this zone, thus giving a safe margin.

Example:

Tube	1	2	3	4	5	6	7
Dilution	1:20	1:30	1:40	1:60	1:80	1:120	1:160
Time in Seconds	18	18	18	18	21	22	24

Dilution used, 1:30. The thromboplastin suspension made up according to the results of the titration may be stored in the icebox and is suitable for use for several days.

#### PREPARATION OF BLOOD

Either venous or capillary blood may be used. For venous blood 100 × 12 mm. tubes are used. The tubes are calibrated to 2.0 c.c., and 0.2 c.c. of M/10 sodium oxalate solution (1.34 Gm. per 100 c.c.) is placed in the tube which is stoppered with a cork. Several of these may be prepared and kept on hand.

For capillary blood 75 × 10 mm. tubes are used. They are calibrated at 0.5 c.c., and 0.05 c.c. of M/10 sodium oxalate solution is placed in each tube. These may be prepared and stoppered.

In taking venous blood about 3 c.c. of blood are drawn into the syringe, and immediately enough is added to the oxalate tube described above to fill it to the 2.0 c.c. calibration mark. The oxalate and blood are mixed by tilting the tube five or six times. In case there is any difficulty in making the puncture a fresh needle and syringe should be obtained and the puncture made at a new site. This oxalated blood may be placed in the icebox for as long as overnight without making any significant change in the prothrombin time.

When blood is desired from an infant, it may be obtained by puncturing the superior longitudinal sinus through the anterior fontanel, obtaining slightly over 2 c.c. of blood. However, our comparative studies have shown that blood taken from a puncture of the heel gives practically the same results as blood taken from the longitudinal sinus. The preferred site of puncture is the edge of the plantar surface of the heel rather than the fat pad in the center of the heel. The site is cleaned with alcohol and the area coated with

vaseline. After making a satisfactory puncture, blood is obtained up to the 0.5 c.c. mark in the tube described above. This should be done with frequent shaking to keep the blood well mixed with the oxalate. Paraffined tubes may be used, but we have found this precaution unnecessary.

When blood is desired from the umbilical cord, it is usually collected from the cut surface, using the tubes calibrated at 2.0 c.c.

The plasma is obtained after centrifugalizing the blood for five minutes at a relatively low speed.

#### TECHNIQUE OF THE PROTHROMBIN TIME DETERMINATION

Thin-walled test tubes, 100 × 12 mm., are used. The tubes are conveniently held in a Wassermann rack. By means of the Folin microsugar pipette 0.1 c.c. amounts of well-mixed thromboplastin suspension are delivered to as many tubes as are needed. With a similar pipette 0.1 c.c. of the plasma is added to the thromboplastin suspension. Each test is done in duplicate. This thromboplastin-plasma mixture may be held at room temperature for as long as an hour, without significant change, but should not be kept in the water bath longer than is necessary for carrying out a small number of tests.

A sufficient amount of M/40 calcium chloride solution (0.365 Gm. calcium chloride crystals per 100 c.c. water) contained in a test tube is placed in a 37° C. water bath. When the thromboplastin-plasma mixture is prepared, it is warmed to 37° C. in the water bath and 0.1 c.c. of the warm calcium chloride solution is added by means of a Folin pipette, at the same instant starting a stop watch. The tube containing the mixture should be kept submerged during this procedure. At the end of fifteen seconds and at every two-second interval after that the tube is momentarily taken from the water bath to be observed for clot formation. This is best seen by tilting the tube, as the clot forms in a rounded mass and slides along the side of the tube. The watch is stopped at the first sign of clot formation.

In some markedly dehydrated newborn infants it may not be possible to obtain sufficient plasma from the 0.5 c.c. blood specimen to run the routine determination. In such cases the whole blood may be used for the test. The same technique is used as for the plasma.

In order to check the system a control determination should be made at the beginning of each set of tests. Either fresh blood from a healthy adult or blood with a known short prothrombin time, which has been kept for not longer than twenty-four hours in an icebox, may be used for this control test.

#### FINDINGS AND DISCUSSION

Our results in the first 100 prothrombin time determinations on normal adults are tabulated in Table I.

The wide variation and high average of the prothrombin time determinations made during the month of February, as compared with the findings in the four following months, suggest that there may be a seasonal variation in the normal level. This study is not extensive enough to make any definite statements, but this point seems worthy of further investigation.

TABLE I  
PROTHROMBIN TIME OF NORMAL ADULTS

MONTH	CASES	LOW	HIGH (TIME IN SECONDS)	AVERAGE
Feb.	28	15	40	28.67
March	21	17	25	20.57
April	10	18	25	21.1
May	31	17	27	21.8
June	10	19	24	22.1
Total	100	15	40	23.4

The average prothrombin time, that is, 23 seconds, is the same as that reported by Quick and co-workers,<sup>4</sup> and also by Butt, Snell, and Osterberg.<sup>5</sup>

As reported by Bray and Kelley,<sup>3</sup> the prothrombin time of cord blood in case of normal infants is usually within the range of normal adults. A prolonged prothrombin time of cord blood may occur in hemorrhagic disease of the newly born infant.

The prothrombin time of the normal newly born infant is very variable. It tends to be low and within normal adult range on the first day, rises to a peak higher than the normal adult range between the second and fifth days, and then comes down within the normal adult range after the fifth day. In hemorrhagic disease of the newborn the prothrombin time may be very high; it may also be high without any evidence of bleeding.

When whole blood is used, the results in normal prothrombin times are practically the same as when plasma is used. In cases of prolonged prothrombin time the whole blood may give a longer time than would result from the use of plasma. However, we do not feel that this is objectionable; in fact the dilution of the prothrombin makes for a more delicate test.

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# HEMOLYTIC EFFECTS OF ETHYL AND CAPRYLIC ALCOHOL\*

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CAPRYLIC alcohol is commonly used to prevent foaming, particularly of blood and other body fluids.<sup>1</sup> Experience has shown that the addition to blood of this substance, even in small quantities, frequently causes hemolysis. This study was designed primarily to determine the hemolytic power of caprylic alcohol. Comparisons were made with water and with ethyl alcohol, both of which are well known hemolytic agents.<sup>2, 3</sup>

## PROCEDURE

Ten samples of fresh, defibrinated ox blood and five specimens of hog's blood were used. The hemolytic agents were added directly to the blood in the following proportions (reagent: blood): for water, 1:10, 1:8, 1:6, 1:4, 1:2, 1:1, 2:1, 3:1; for ethyl alcohol, 1:25, 1:10, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1; for caprylic alcohol, 1:1,000, 1:600, 1:500, 1:400, 1:300, 1:200, 1:100, 1:50, 1:25, 1:10. Mixtures showing perceptible darkening, extreme darkening, transparency, and (for the alcohol-blood mixtures) hemolysis in 0.9 per cent sodium chloride solution were noted at intervals of five, fifteen, thirty, forty-five, and sixty minutes. Thorough mixing by gently inverting the tubes preceded each observation.

The various criteria of hemolysis were judged in the following manner. *Perceptible darkening* was determined by comparison with a tube of untreated blood from the same general sample. *Extreme darkening* indicated the plum color characteristic of laked blood. *Transparency* denoted ability to see print clearly through the blood film left by inversion of the test tube. *Hemolysis in 0.9 per cent sodium chloride solution* was tested by transferring a small amount of each blood mixture to a tube containing 5 c.c. of the salt solution.

Resistance to hypotonic laking of red cells from mixtures not completely hemolyzed after one hour was tested by transferring small amounts to sodium chloride solutions having concentrations of 0.42 to 0.90 per cent.

All observations were made at room temperature. The average was 23.9° C., with extremes at 21.5° and 28.0°; comparisons were made at equal temperatures.

## RESULTS

Results are summarized in the accompanying tables. Table I shows the dilution of hemolytic agent in blood at which the various criteria of hemolysis appeared in one hour, together with the extremes encountered and the percentage of samples having the median value. Considerable variation occurred with regard to perceptible darkening, which represents the earliest detectable

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stage of hemolysis. Extreme darkening and transparency, indicating virtually complete hemolysis, gave more uniform results. According to the latter criteria, ethyl alcohol showed approximately four times the laking power of water, while caprylic alcohol was fifty times as powerful as ethyl alcohol and two hundred times as powerful as water. Hemolysis in 0.9 per cent sodium chloride solution was recorded only for mixtures containing the alcohols. Results were practically the same as those for transparency. Some slight differences between ox blood and hog's blood are shown in Table I.

TABLE I  
DILUTIONS OF WATER, ETHYL ALCOHOL, AND CAPRYLIC ALCOHOL AT WHICH  
VARIOUS CRITERIA OF HEMOLYSIS APPEAR

CRITERIA OF HEMOLYSIS	WATER			ETHYL ALCOHOL			CAPRYLIC ALCOHOL		
	MEDIAN		EX- TREMES	MEDIAN		EX- TREMES	MEDIAN		EX- TREMES
	Dil.	Per Cent		Dil.	Per Cent		Dil.	Per Cent	
<i>Ox blood</i>									
Perceptible darkening	6	80	4,6	10	100		400	20	200,600
Extreme darkening	1	90	1,2	4	80	4,5	200	80	100,200
Transparency	1	80	1,2	4	100		200	50	100,200
Hemolysis in 0.9% NaCl				4	100		200	70	200,200
<i>Hog's blood</i>									
Perceptible darkening	2	80	2,4	6	100		300	60	300,400
Extreme darkening	1	80	1,2	5	60	4,5	200	100	
Transparency	1	100		4	80	4,5	200	100	
Hemolysis in 0.9% NaCl				4	100		200	100	

Notes: Figures in "dilution" columns represent parts of blood per one part of reagent in the test mixture. Figures in "per cent" columns refer to the percentage of samples having the median value.

While caprylic alcohol was a much more powerful hemolytic agent than ethyl alcohol or water, it was slower in developing its maximal effect. The maximum hemolytic effect of water was reached in five minutes and that of ethyl alcohol in less than fifteen minutes. Caprylic alcohol, on the other hand, caused laking in progressively weaker dilutions during one hour and may not have reached its end point in that time.

The osmotic resistance of cells from mixtures not completely hemolyzed after one hour is shown in Table II, which shows also the direction of change from the control value for individual samples. The recorded figure for osmotic resistance represents in each case the midpoint of the zone between complete hemolysis and imperceptible hemolysis. As only large differences were of interest, no attempt was made to measure fragility of red blood cells with great accuracy. None of the water-blood mixtures showed important alterations in osmotic resistance. In every instance, mixture of ethyl alcohol with blood in proportions of 1:5 caused a large decrease in resistance to hypotonic laking, while 1:10 dilutions resulted in a definite decrease in but one sample out of thirteen. In the case of caprylic alcohol, the great majority of 1:200 mixtures were completely laked, while 1:300 and 1:400 dilutions uniformly exhibited large decreases in osmotic resistance. Smaller but still definite decreases were ob-

served in a majority of 1:500 dilutions with ox blood, and a few even in dilutions of 1:600. Again differences between ox and hog's blood were noted but were considered of doubtful significance.

TABLE II

OSMOTIC RESISTANCE OF RED BLOOD CELLS EXPOSED TO VARIOUS HEMOLYTIC AGENTS FOR ONE HOUR

HEMOLYTIC AGENT	DILUTION	OSMOTIC RESISTANCE			CHANGE IN OSMOTIC RESISTANCE					
		EX- TREMES	AVG.	MED.	DECREASE		INCREASE		NO CHANGE	
		Per Cent NaCl	Per Cent	Per Cent	Num-ber	Per Cent	Num-ber	Per Cent	Num-ber	Per Cent
<i>Ox blood</i>										
Control		0.43-0.52	0.48	0.49						
Water	1	0.43-0.45	0.44	0.44	0	0.0	0	0.0	2	100.0
	2	0.47-0.52	0.49	0.49	0	0.0	0	0.0	8	100.0
Ethyl alcohol	5	0.53-0.85	0.60	0.65	10	100.0	0	0.0	0	0.0
	10	0.46-0.54	0.50	0.50	0	0.0	0	0.0	9	100.0
Caprylic alcohol	300	0.55-0.85	0.72	0.75	7	100.0	0	0.0	0	0.0
	400	0.51-0.85	0.65	0.65	9	100.0	0	0.0	0	0.0
	500	0.47-0.65	0.59	0.60	7	87.5	0	0.0	1	12.5
	600	0.43-0.65	0.53	0.53	3	50.0	0	0.0	3	50.0
<i>Hog's blood</i>										
Control		0.46-0.49	0.47	0.48						
Water	2	0.46-0.49	0.47	0.48	0	0.0	0	0.0	5	100.0
Ethyl alcohol	5	0.65-0.85	0.80	0.76	5	100.0	0	0.0	0	0.0
	6	0.50-0.60	0.58	0.55	3	60.0	0	0.0	2	40.0
	10	0.44-0.58	0.47	0.49	1	25.0	0	0.0	3	75.0
Caprylic alcohol	300	0.80-0.90	0.85	0.85	5	100.0	0	0.0	0	0.0
	400	0.51-0.60	0.57	0.57	5	100.0	0	0.0	0	0.0
	500	0.46-0.52	0.49	0.49	0	0.0	0	0.0	5	100.0
	600	0.47-0.52	0.48	0.49	0	0.0	0	0.0	5	100.0

Notes. Figures in "dilution" column represent parts of blood per one part of reagent in the test mixture. Only samples showing alteration in osmotic resistance in excess of 0.04 per cent sodium chloride are considered as changed in this table.

The results indicate also the proportions of these reagents which may be added to blood without gross hemolysis in one hour. Water failed to produce perceptible darkening in dilutions of 1:8, while 1:6 dilutions showed this in 80 per cent of the ox blood samples. Extreme darkening and transparency never appeared in dilutions of 1:4 but were almost the rule in 1:1 mixtures. Ethyl alcohol uniformly caused some hemolysis in 1:10 mixtures with ox blood but never in 1:25 dilutions. Extreme darkening and transparency failed to appear at 1:6 but were rather frequent at 1:5. With caprylic alcohol, perceptible darkening was never observed in dilutions of 1:1,000 and appeared only once at 1:600. It occurred at 1:500 in 30 per cent, at 1:400 in 20 per cent, and at 1:300 in another 30 per cent of the ox blood mixtures. Extreme darkening never appeared at 1:400 and only once at 1:300. Eighty per cent of the ox blood and all of the hog's blood samples showed extreme darkening at 1:200. Transparency did not develop in dilutions of 1:300 but was present in half of the ox blood and all of the hog's blood samples at 1:200.

## SUMMARY

1. The relative hemolytic powers of water, ethyl alcohol, and caprylic alcohol were tested at room temperature over a period of one hour by mixing the hemolytic agents with defibrinated blood in various proportions.

2. Water caused hemolysis rather uniformly in dilutions of 1:1. Ethyl alcohol was found to have approximately four times the laking power of water. Caprylic alcohol had about fifty times the laking power of ethyl alcohol and two hundred times that of water.

3. Resistance to hypotonic laking of cells from mixtures not completely hemolyzed in one hour was unchanged when water was the hemolytic agent, and decreased over a limited range in the case of ethyl alcohol-blood and caprylic alcohol-blood mixtures.

4. The following dilutions of reagent in blood seemed practically devoid of gross hemolytic effects in one hour: for water, 1:8; for ethyl alcohol, 1:25; for caprylic alcohol, 1:600.

It is a pleasure to acknowledge the helpful suggestions of Dr. C. C. Guthrie and Dr. T. K. Kruse.

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### THE LANGE TEST\*

#### II. THE INFLUENCE OF PARTICLE SIZE AND HYDROGEN-ION CONCENTRATION OF GOLD SOLS UPON LANGE TEST READINGS ON SYPHILITIC AND TABETIC SPINAL FLUIDS

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EXPERIMENTAL results summarized in a previous paper<sup>1</sup> indicate that the sensitivity of gold sols in the Lange test for paresis increases with increase in particle size and decreases with increase in pH. The present paper summarizes the results of a similar study with syphilitic and tabetic spinal fluids. The gold sols used were prepared by the nuclear formaldehyde method in exactly the same manner as in the study with paretic fluids, and all techniques were carried out as previously described.

TABLE I  
INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "B"	pH OF SOLS 8.3
Average particle size in millimicrons	Test readings
43	3344443321
40	2233321000
38	1223310000
36	1223211000
23.5	1223100000

\*From the Department of Chemistry, University of Wisconsin, Madison.  
Received for publication, May 25, 1939.

The results obtained are summarized in Tables I to XV. The eleven sets of syphilitic data were selected at random from a large amount of similar data. The four sets of tabetic data represent all the information obtained on tabetic fluids.

TABLE II  
INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "C"	pH OF SOLS 6.99
Average particle size in millimicrons	Test readings
43	3332100000
41	1233100000
39.5	1121100000
38	1110000000

TABLE III  
INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "F"	pH OF SOLS 6.6
Average particle size in millimicrons	Test readings
43	4332100000
41	2233210000
39.5	1122100000
38	1221000000

TABLE IV  
INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "D"	pH OF SOLS 6.0
Average particle size in millimicrons	Test readings
45	3333210000
43.5	2333210000
38	2233100000
35	1222100000

TABLE V  
INFLUENCE OF PARTICLE SIZE ON SYPHILITIC READINGS

SYPHILITIC FLUID "G"	pH OF SOLS 7.4
Average particle size in millimicrons	Test readings
43	2333100000
41	1221000000
39.5	1100000000
38	0000000000

TABLE VI  
INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "H"	PARTICLE SIZE 43 MILLIMICRONS
pH	Test readings
7.38	2333100000
7.01	3333100000
6.62	4332100000
6.10	4433210000



TABLE VII  
INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "J"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
7.57	1111000000
7.22	1123100000
6.97	1233100000
6.65	2233210000

TABLE VIII  
INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "K"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
7.57	1232100000
7.22	1333100000
6.97	2332200000
6.65	2333210000
6.4	3333220000

TABLE IX  
INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "L"	PARTICLE SIZE 39.5 MILLIMICRONS
pH	Test readings
7.46	1100000000
6.94	1121100000
6.61	1122100000
6.41	2232100000
6.22	2233210000

TABLE X  
INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "N"	PARTICLE SIZE 38 MILLIMICRONS
pH	Test readings
6.99	1110000000
6.50	1122100000
6.18	2232100000
5.60	2233110000

TABLE XI  
INFLUENCE OF pH ON SYPHILITIC READINGS

SYPHILITIC FLUID "M"	PARTICLE SIZE 38 MILLIMICRONS
pH	Test readings
8.3	1122210000
8.0	1234311000
7.4	2244321000
6.6	2344431100
6.2	2454442100

TABLE XII  
INFLUENCE OF PARTICLE SIZE ON TABETIC READINGS

TABETIC FLUID "A"	pH OF SOLS 7.90
Average particle size in millimicrons	Test readings
42	3444432100
41	2344421000
36	2334421000
30	1233410000
23.5	1122210000

TABLE XIII  
INFLUENCE OF PARTICLE SIZE ON TABETIC READINGS

TABETIC FLUID "B"	pH OF SOLS 7.30
Average particle size in millimicrons	Test readings
42	5555543210
41	5555543100
36	3444310000
30	2234310000
23.5	1233310000

TABLE XIV  
INFLUENCE OF pH ON TABETIC READINGS

TABETIC FLUID "A"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
7.90	2344421000
7.50	3444431000
7.28	3455432100
6.60	5555541100
6.00	555554211

TABLE XV  
INFLUENCE OF pH ON TABETIC READINGS

TABETIC FLUID "B"	PARTICLE SIZE 41 MILLIMICRONS
pH	Test readings
8.20	2343220000
7.80	3444331000
7.50	4455543210
7.30	5555543100
6.60	555553110
6.00	555555422

#### SUMMARY

The results indicate that, as in the case of paresis so in the case of syphilis, the sensitivity of gold sols in the Lange spinal fluid test increases with increase in particle size and decreases with increase in pH. Although only a limited amount of data is available on tabetic fluids, the results obtained indicate that these fluids respond to changes in pH and particle size of gold sols as do parietic and syphilitic fluids.

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1. Glasoe, P. K., and Sorum, C. H.: The Lange Test. I. The Influence of Particle Size and Hydrogen-Ion Concentration of Gold Sols Upon Lange Test Readings on Parietic Spinal Fluids, J. LAB. & CLIN. MED. 25: 1, 1939.

# FUNGICIDAL TESTING: A COMPARISON OF METHODS\*

ADELIA MCCREA, PH.D., DETROIT, MICH.

IN A RECENT issue of Drug Trade News,<sup>1</sup> considerable discussion was given to three methods for making fungicidal tests: those advocated by Reddish,<sup>2</sup> by Klarmann,<sup>3</sup> and by Taft.<sup>4</sup> It is desired to comment herein upon these tests and to suggest a method which shows more clearly the power of a vigorous fungus to survive adverse conditions.

In an earlier paper<sup>5</sup> a method which has proved useful in practical testing of fungicidal agents offered for clinical purposes was outlined briefly. This basic method has now been followed for years in our laboratories and has, naturally, been subject to slight modifications in response to continued experience. It seems advisable to report such minor changes and to review the evidence favorable for this method.

In any such test there are two outstanding factors: the fungus and the test substance. Other factors are variable according to conditions; but these two must be definite, repeatable, and reasonably standardized, if results obtained in different laboratories are to be fairly evaluated.

One major point to keep in mind is that fungi of mold type are not closely comparable to bacteria and cannot be handled successfully by the same methods. A mold consists of threadlike parts (hyphae), more or less intricately woven together into a mat of mycelium; and of fruiting portions with a great variety of spores which are comparable to the seeds of higher plants. Some of the hyphae are feeders which by their rootlike parts (rhizoids) can penetrate the substratum and absorb food for the whole plant. Other hyphae (sporophores) grow upright to bear specialized fruiting branches upon which the spores are developed. In fact, a mold more nearly resembles a shrub or tree than it does a bacterium, so far as *structure* is concerned.

A single species of fungus may reproduce by three or four different types of spores, as well as by "slips" or "cuttings" of the mycelium. Any properly made suspension of mold growth will contain not only several types of spores characteristic of the species but also fragments of hyphae, any intact cell of which may form a new plant. Generally speaking, spores are more resistant to fungicidal agents than are the hyphal cells. Clearly, then, a fungous culture which is to be used in a test must be *allowed to grow under favorable conditions for a sufficient time to permit it to develop a typical mature structure.*

Like the higher plants, fungi vary in the length of time required to mature, i.e., to "ripen" their spores. For *Monilia* and related types, forty-eight to seventy-two hours is sufficient. For many species of *Aspergillus*, *Penicillium*,

\*From the Parke, Davis and Co. Research Laboratories, Detroit.  
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etc., seventy-two to ninety-six hours will suffice; but those species which produce *ascospores* should grow ten days to two weeks before being used in suspensions. Among the true dermatophytes—species of *Trichophyton*, *Epidermophyton*, *Microsporum*, *Achorion*—two weeks is the shortest, four weeks the longest, period of growth permissible if a fair test is to be made. This insures that a normal mature growth of the fungus in a vigorous state with characteristic spores will be pitted against the test substance. Some species of these genera scarcely begin to form their macrospores ("fuseaux") in five days, which is the growth period set by the Reddish method.<sup>2</sup> To use an immature five-day culture of such a species will give an erroneously high value to the fungicide being tested because such young mycelium is more easily killed.

The glass bead method advocated by Klarmann is also open to criticism. Aside from a few "water molds," parasitic on aquatic animals and plants, there are not many filamentous fungi which develop normally in a liquid medium. Although beads offer a platform upon which the fungous mat may rest, the hyphae cannot penetrate to obtain a firm hold. Shaking such relatively weak growth with the beads to form a suspension is a questionable practice. Hyphal walls and spore cells are ruptured, and the resultant suspension deteriorates before the real test begins. It is as if one were to take a geranium cutting, bruise it badly until the cells were broken and their contents oozing out, then pot it and expect a normal plant. In this connection, attention should be directed to a misinterpretation of my earlier study<sup>3</sup> by Burlingame and Reddish.<sup>6</sup> *At no time have I advocated the use of glass beads in the preparation of either growth or suspension for testing.*

In my experience the agar cup plate method has proved unsatisfactory for mycologic work, except to give confirmatory evidence of the results obtained by the test tube method, and for testing ointments or gels. The relation is negligible between a soft agar jelly and the horny, keratinous tissues of skin, nails, and hair which are the natural habitat of dermatophytic fungi. These pathogens do not penetrate into the deeper layers of the skin, nor into other tissues beneath it. Scars are not left from infection. If penetration must be included in a fungicidal test (which I am not ready to concede), then it should be studied through the use of fungi growing upon horn scrapings, nail clippings, hair, or skin. Although possible, this is scarcely practical because of the time usually required for such cultures to attain suitable growth—about six to eight weeks.

These fungi are strongly *aerobic*. (Even the fungi which infect internal organs, as the lungs, are harmful quite as much because of occlusion of the air spaces as because of actual attack upon the tissues.) *Temperature* is another important factor. Few species will grow well and many are killed if kept at blood temperature. (Our cultures are grown at 27° to 32° C. During hot weather they are placed in the refrigerator to retain their vitality.) Consequently, Taft's<sup>4</sup> delayed cup plate test is inconsistent with the nature of these organisms. The fungus is both overheated and smothered before one proceeds with the test.

The ideal method would be to test fungicides against actual conditions on patients. As this is not feasible, the next best way might seem to be the production and treatment of lesions on animals. This would be my method of choice except that it is limited to the species which are parasitic upon lower animals, hence does not include all the fungi pathogenic for human beings. However, we make considerable use of it wherever possible.

Details of the method I employ are outlined briefly below. The formula for Sabouraud's medium, the species of fungi used, and other explicit information are to be found in a previous report.<sup>5</sup> In addition to the two species mentioned, *Trichophyton interdigitale* Priestley and *Epidermophyton rubrum* Cast., two other dermatophytes are usually tested, *Achorion gypseum* Bodin and *Microsporum lanosum* Sab. Frequently *Aspergillus niger* Tieghem is included when information is desired as to the effect of the test substance on laboratory weeds.

I. Spore Suspension. Here is where the crux of the test really lies. A poorly prepared, deteriorated suspension is much easier to kill than one which contains the spores and mycelial elements of a normally vigorous, sporulating fungus.

In growing the cultures of skin fungi for test purposes, test tubes 18 by 140 mm. are used, with 8 c.c. of Sabouraud's glucose agar, slanted to leave a butt of about one-half inch, and planted while fresh so that growth will cover the entire surface. (Equivalent surface in flask cultures would, of course, do as well.) When mature growth has occurred, as described above, suspension is prepared by washing the surface with sterile, distilled water. For such species as *Monilia* and related forms, 10 c.c. per tube are used; for such cultures as *Aspergillus* and *Penicillium*, 12 c.c. per tube; for dermatophytic fungi, 8 c.c. per tube. The underlying medium should never be broken, but spores may sometimes need to be dislodged by gentle strokes of a bent wire or a loop.

Spores and mycelial fragments are prone to "clump" in groups, hence the suspension should be strained through sterile cloth—double layer, if gauze; single, if mull is used. This gives a homogeneous suspension with microspores (conidia), macrospores ("fuseaux"), and mycelial cells throughout the liquid. Because of these multiple elements in a fungous suspension, I have not found a counting chamber of value, and rely upon the growth obtained from a comparable area of a thrifty culture at a definite age to assure a comparable suspension; I believe this to be as nearly exact as one can reach in handling living materials. Control tubes are planted at the beginning and end of each dilution tested.

II. Test Substance. Dilution Series. There is no magic formula for a dilution series. The usual Food and Drug Administration method is readily applicable, except that planting should be made upon agar medium instead of liquid. In most cases, a preliminary, wide dilution series will set the limit for the final series of dilutions.

III. Exposure Intervals. We use one, five, ten minutes, and one hour exposure intervals. When *fungistatic* effect is to be studied, daily transplants are made for any desired period. We customarily allow three weeks.

IV. Incubation Period. For short life-cycle fungi, as *Monilia*, *Cryptococcus*, etc., final records may be taken seventy-two hours after planting.

For longer life-cycle species, as the dermatophytes, the *Aspergilli*, the *Penicillia*, etc., records should not be considered complete until at least one week after testing. Germination often may be *delayed* a few days, with a good growth appearing later.

For practical purposes, no other method yet devised is as satisfactory as this *in vitro* (test tube) method to give comparative criteria on liquid fungicidal agents. Its outstanding advantages are:

1. It uses only thrifty, mature cultures with their spores well developed as they are found in nature.

2. After the suspension has been properly prepared, this method is closely comparable to the well-known official method of testing germicides. (F.D.A., with or without the Shippen modification.) Adequate controls aid in correct interpretation of results.

3. It gives ample scope for individual choice of dilution series and time intervals.

4. Its results can be check-tested without much delay, provided a sufficient number of cultures was prepared in the original planting. In laboratories where much testing is carried on, cultures may be planted routinely at two- to three-week intervals so that they are available at all times in a suitable stage of development.

Bacteriologists, chemists, and general technicians should realize the necessity to consider the *mycologic nature* of species which they use in any fungicidal test. True data and fair comparisons are not possible unless this is done.

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6. Burlingame, Ella M., and Reddish, George F.: Laboratory Methods for Testing Fungicides Used in the Treatment of Epidermophytosis, J. LAB. & CLIN. MED. 24: 765, 1939.

# THE CENTRIFUGE TECHNIQUE IN THE HETEROPHILE AGGLUTINATION TEST\*

ALVIN HOLLANDER, B.S., M.S., BROOKLYN, N. Y.

CENTRIFUGATION offers a rapid and reliable method for agglutination tests. It was first established as a routine procedure by Schiff<sup>1</sup> in his monograph on blood grouping. The results of heterophile agglutination tests performed by the centrifuge method are here presented.

The test is done in Wassermann tubes, 1.3 cm. wide by 10.3 cm. long. Inactivated serum is diluted serially in steps of one-half and added to the tubes in a volume of 0.5 c.c. One-half cubic centimeter of a 2 per cent suspension of washed sheep cells which have been collected twenty-four hours previously is then added. The mixtures are centrifuged for five minutes at about 1,000 r.p.m., and are then shaken and read for agglutination. Table I compares 98 various sera and 5 sera from cases of infectious mononucleosis examined by the Paul-Bunnell and the centrifuge techniques.

TABLE I

POSITIVE IN DILUTION OF SERUM	WASSERMANN SERA		INFECTIOUS MONONUCLEOSIS	
	CENTRIFUGE METHOD*	PAUL-BUNNELL METHOD*	CENTRIFUGE METHOD	PAUL-BUNNELL METHOD
1:2	27	28		
1:4	23	26		
1:8	22	26		
1:16	13	6		
1:32	2	4		
1:64	5	2		
1:128	1			
1:256			1	1
1:512			1	2
1:1,024			1	1
1:2,048			2	1

\*No agglutination was observed with either the centrifuge or the Paul-Bunnell method in 5 and 6 sera, respectively.

It is seen from the table that with the centrifuge method there is a shift toward agglutination with higher dilutions of serum, indicating the greater sensitivity of this reaction. In no case, however, was a difference of more than one dilution observed. The rapidity with which the heterophile agglutination test can be completed by means of centrifugation gives it a distinct advantage over the Paul-Bunnell technique.

## REFERENCE

1. Schiff, F.: Die Technik der Blutgruppenuntersuchung, ed. 3, Berlin, 1932, Julius Springer.

\*From the Division of Bacteriology, Jewish Hospital of Brooklyn.  
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## THE APPLICATION OF AN ENDOSPORE STAIN TO BLOOD SMEARS FROM OPSONOPHAGOCYTIC TESTS\*

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IN ORDER to obtain an accurate count of the bacteria phagocytized in an opsonophagocytic test, it is necessary to employ a stain which will cause the engulfed organisms to stand out distinctly within the cytoplasmic substance of the leucocytes. Recently, some difficulty was encountered in staining a *Brucella* strain of equine origin in blood smears made from opsonophagocytic tests. Wright's stain, although producing excellent results with the blood cells, did not color the bacteria intensely enough for an accurate count. Hastings' stain gave more deeply colored microorganisms, but the contrast between the bacteria and the phagocytes was not as striking as we wished for our tests. Since the organisms were gram-negative, the Gram stain was of no value. Other attempts failed to produce the desired differential staining until a modified form of the Schaeffer and Fulton<sup>1</sup> endospore stain was used.

Schaeffer and Fulton<sup>1</sup> simplified the endospore staining technique of Wirtz<sup>2</sup> by eliminating fixing in osmic acid, shortening the time of staining, and substituting aqueous safranine for dilute carbolfuchsin. The procedure as evolved by them consisted of the application of a 5 per cent aqueous malachite green solution to smears fixed by flaming. This stain was heated and allowed to stand for one-half minute. It was then washed off with tap water after which 0.5 per cent aqueous safranine was added as decolorizer and counterstain. The spores were stained green and the cells red. By allowing the malachite green to act for a longer interval without heating and then applying safranine for a shorter period, we obtained a preparation in which the bacteria were stained green, the nuclei of the leucocytes a deep red, and the cytoplasm a light pink. There was no blending of the colors and phagocytized microorganisms stood out clearly as definite green bodies within the pink cytoplasm of the white blood cells.

The modified technique which gave the best results is as follows:

1. Make a smear from the test material by spreading the contents of a 2 mm. loop over an area of 4 sq. cm., air dry, and fix by flaming three times.

\*From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.

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2. Apply 5 per cent aqueous malachite green solution for 5 minutes.
3. Wash for 10 to 20 seconds with tap water.
4. Apply 0.5 per cent aqueous safranin solution for ten seconds.
5. Wash quickly with tap water, dry, and examine.

Heat fixation of a fairly heavy smear produced a preparation free from intact erythrocytes and at the same time retained a sufficient number of leucocytes within a limited area. Other differential stains were tried on similar slides, but in no case was the contrast between the bacteria and the white blood cells as marked as in the malachite green-safranin combination.

The technique is not applicable to all species of bacteria for some strains are unable to retain the malachite green stain in the presence of safranin. It was noted that staphylococci are stained red by this method whereas streptococci present a few green cells in a field occupied for the most part by red cocci. Some gram-negative bacteria are stained green and others become red under similar treatment.

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2. Wirtz, R.: Eine einfache Art der Sporenfärbung, *Centralbl. f. Bakt. Abt. I (orig.)* 46: 727, 1908.

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### A NEW TUBE CLAMP\*

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MOHR'S pinchcock is probably the one most widely used because it can be so quickly adjusted to the tubing, and because it is simple in design and low in cost. The only disadvantage of this pinchcock is that it can be adjusted to the tubing only by sliding it on from the end, and this sometimes involves disconnecting apparatus. To overcome this difficulty I have designed a pinchcock from two Mohr's pinchcocks, by cutting off from each, one jaw with its finger press, leaving about 16 mm. more jaw on one clamp than on the other. Holes are then cut for a bolt, the head of which is countersunk in what forms the inside surface of the jaw of the new clamp. The bolt is secured by a nut on the outside. This allows one finger press to be moved through a right angle, enabling a piece of rubber tubing to be taken into the clamp, or released from it, without having to slide the clamp over the end of the tubing.

The principle can be seen at a glance in the photographs of Fig. 1.

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\*From the Department of Pharmacology, University of Texas School of Medicine, Galveston.

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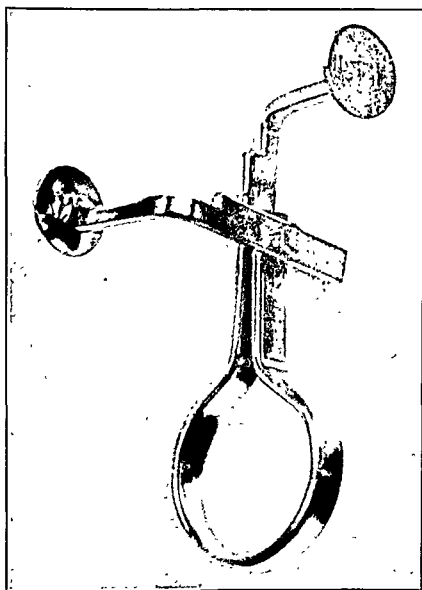
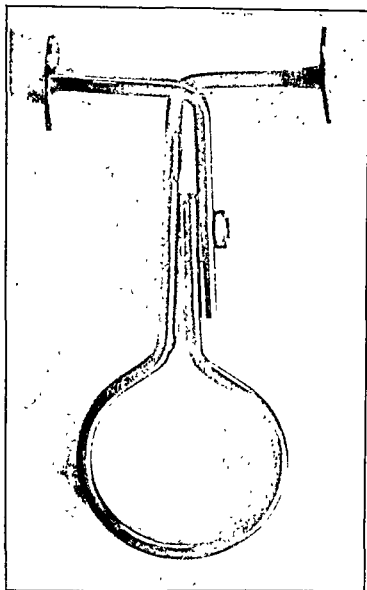


FIG. 1.—Modified Mohr's pinchcock. *Upper*, modification of ordinary type; *Lower*, finger press moved through a right angle.

# A METHOD OF DESTROYING THE BLASTOCYSTS (BLASTOCYSTIS HOMINIS) IN FECAL WET SMEARS IN ORDER TO FACILITATE THE EXAMINATION FOR ENDAMOEBIA HISTOLYTICA\*

E. G. HAKANSSON, M.D., WASHINGTON, D. C.

IN ALMOST all texts dealing with the examination of feces for intestinal protozoa the reader is warned against blastocysts. Rightly so, because the beginner and even the laboratory worker with some experience frequently have trouble in differentiating the blastocysts from the cysts and rounded up trophozoites of *Endamoeba histolytica* and from some of the other intestinal protozoa. Undoubtedly not a few persons who harbor this innocent vegetable organism have been told that they have amoebic dysentery. We have seen this happen in a few instances.

There is a simple and practical way to prevent such mistakes. The blastocysts when placed in tap water or distilled water distend, rupture, and disintegrate as a result of endosmosis. The trophozoites of the intestinal protozoa are similarly destroyed, but the cysts withstand the decreased osmotic pressure and remain unchanged for many hours. Thus, if a fecal smear is made in water instead of the usual normal saline solution, blastocysts and protozoan trophozoites disappear, leaving a clear field for any cysts that may be present.

The practical significance of this should be obvious to those who labor with examinations of feces. With very few exceptions the identification of *E. histolytica* in clinical laboratories is made, or should be made, on the cysts. The search for cysts is usually made in a normal saline smear. Low magnification, for example 16 mm. objective and 7.5 ocular, has to be used in order to cover an adequate area within a reasonable time. With this magnification the blastocysts and the protozoan cysts, as well as the smaller trophozoites, usually appear so similar that they cannot with certainty be set apart. Whenever blastocysts are present, and they are present in the great majority of stools, it, therefore, becomes necessary to search with the 4 mm. objective. Few workers in clinical laboratories will have time for covering even a single smear with this high magnification. Furthermore, some forms of blastocysts resemble the protozoan cysts so closely that even with the 4 mm. objective each individual has to be scrutinized, all of which requires much time. A smear in tap water eliminates this nuisance. By the time the smear has been made and the preparation is ready to be examined the blastocysts have either disappeared completely or become so distended and lusterless that they readily can be differentiated from the shining refractile cysts.

\*From the U. S. Naval Medical School, Washington, D. C.  
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It should be mentioned that if a large particle of feces is used in making the aqueous smear, salts in the feces may dissolve in the water to such an extent as to make the smear almost isotonic and thus defeat the purpose of the smear. It is well, therefore, to pick up only the small particle of feces needed for the smear and avoid thick smears.

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## THE CAPILLARY HEMATOCRIT METHOD OF DETERMINING BLOOD CELL VOLUME\*

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CHRISTOPHER J. HAMRE, PH D., HONOLULU, T. H.

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### INTRODUCTION

**I**N UNDERTAKING an extensive survey of cellular and hemoglobin blood levels of University students and pre-school children, the problem of including cell volume determinations that did not necessitate securing the blood sample by venipuncture presented itself. A search of the literature disclosed several methods requiring small quantities of capillary blood. Since many methods have been criticized for their accuracy because they require dilution of the blood with an anticoagulant, an attempt was made to develop a technique which would not necessitate dilution of the sample. Several such methods have been described, among which are those of Daland,<sup>1</sup> Capps,<sup>2</sup> Epstein,<sup>3</sup> Rosahn,<sup>4</sup> Miller,<sup>5</sup> and Gram and Norgaard.<sup>6</sup> Of these, the technique and apparatus described by Rosahn seemed to be the most practical from the point of view of ease of manipulation and application to our work.

### DESCRIPTION OF PIPETTES

Thick-walled glass tubing, possessing a uniform capillary bore, in sizes which varied in diameter of bore from 0.5 to 1.8 mm., were cut into 11 cm. lengths for fashioning into capillary pipettes. One end of the capillary pipettes was ground and fashioned to receive a rubber suction tube while the other end was ground to form a long bevel of 8 to 10 mm., the tip of the pipette being left at a diameter of 1.5 to 2 mm. The pipettes were closed by encircling them from end to end with rubber bands  $3\frac{1}{2}$  inches long and  $\frac{3}{4}$  inch wide. Rubber bands narrower than  $\frac{3}{4}$  inch would not stay in place while the pipettes were being centrifuged. Rubber bands alone were soon cut by the pipettes during centrifuging but rubber bands reinforced with automobile tire inner tube patches could be used in several hundred determinations. A short, wide-beveled pipette could not be properly closed with the reinforced rubber bands, a small but significant quantity of blood being lost into the small space formed between the rubber band and the tip of the pipette. The longer bevel described above resulted in effective closure of the pipette by eliminating

\*From the Department of Zoology, the University of Hawaii, and the Nutrition Laboratory of the Hawaii Agricultural Experiment Station, Honolulu, T. H.

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this space. After centrifuging blood samples in these pipettes, the total blood columns and the red blood cell columns were measured on a millimeter ruler and the volumes of the red blood cells determined as per cent of the total volume of blood.

#### METHOD OF USE OF PIPETTES

A series of comparative studies were made to determine the accuracy of pipettes differing in diameter of bore. In one series of studies the pipettes were filled with venous blood prepared for cell volume determinations by the Wintrobe method,<sup>7, 8</sup> determinations using both the Wintrobe tubes and capillary tubes being carried out simultaneously on the same sample of blood. In another series of studies the capillary pipettes were filled with capillary blood for comparison with Wintrobe determinations of cell volume on venous blood of the same individual. Pipettes with bores of 0.5, 0.6, 0.8, 1.0, 1.2, 1.3, and 1.8 mm. in diameter were used in these studies. It was found that the pipettes of smallest bore frequently showed cell volumes 0.5 to 1 per cent lower than those obtained with the larger pipettes. Determinations made with the three largest pipettes varied 0.5 per cent higher or lower than determinations of the cell volume on the venous blood of the same individual by the Wintrobe method. The pipettes of 0.8 and 1.0 mm. bores were found to be accurate and were adopted for use. In this series of studies and in all subsequent studies the pipettes were centrifuged until a constant cell volume was obtained, fifteen minutes being required for the angle centrifuge (3,800 r.p.m.) and thirty minutes for the horizontal type of centrifuge (3,200 r.p.m.).

*Blood Not Mixed With Anticoagulant.*—Though fairly good packing of the red blood cells was secured with clean pipettes centrifuged immediately after being filled with capillary blood, it was clearly evident that coating the pipettes with a small quantity of anticoagulant which would not significantly alter the results by dilution of the blood sample would be desirable. As sodium citrate gave the best initial results, further experiments were conducted to learn its most effective concentration and method of use. Saturated solutions were diluted to make one-fourth, one-third, one-half, two-thirds, and three-fourths saturated solutions. The higher concentrations produced a significant amount of shrinkage while the one-fourth saturated solution did not give consistent results. The one-third saturated solution was consistently effective and, when properly used, did not produce significant changes through shrinkage or dilution. In practice, the one-third saturated solution was drawn into the pipette, the excess expelled, and the pipette partially dried by blowing through it. If the pipettes were centrifuged immediately after being filled, the cells packed completely. In this series of studies it was learned that fungi grew rapidly in the one-third saturated citrate solutions and that when solutions containing fungi were used packing of the cells could not be secured. They did not grow, however, in saturated citrate solutions, and the practice of making up the dilute solutions just before they were to be used was therefore adopted.

Though the method of using sodium citrate, as described above, yielded consistently accurate results, other anticoagulants were also tried. The ox-

alates and oxalate mixtures did not always yield accurate packing of the cells. If they acted effectively as anticoagulants, they usually produced significant shrinkage of the cells. Heparin used as a 1 per cent solution in the same manner as the one-third saturated sodium citrate solution proved effective, yielding the same results as the citrate solution.

None of the anticoagulants used for wetting the pipettes before filling them with capillary blood in the manner described above was effective for more than a few minutes after the pipettes had been filled, poor packing of the cells resulting if the pipettes were allowed to stand for a short time before being centrifuged. Wetting the pipettes with the anticoagulants, including heparin, and drying them completely before filling them with blood was also ineffective. In all cases the failure of the cells to pack after delayed centrifuging was due to the formation of coagula in the center of the pipette and in some limited areas along the side of the pipette, pointing to incomplete mixture of the blood and anticoagulants as the direct cause of failure of the cells to pack.

*Blood Samples Mixed With Anticoagulants.*—Since mixing of the blood and minute quantities of dry anticoagulants could not be effected in the pipettes and delayed centrifuging of the blood samples was sometimes necessary, mixing of the blood and dry anticoagulants before filling the pipettes was attempted as a means of obtaining accurate results when centrifuging was delayed. Guest and Siller,<sup>9</sup> Kato,<sup>10</sup> Smith,<sup>11</sup> Andresen,<sup>12</sup> and Rogatz<sup>13</sup> have described methods of mixing small quantities of blood and anticoagulants. The method of using a depression slide for receiving the blood and mixing it with dry anticoagulants, as described by Kato, appeared to be the best suited to our work. The depression (15 mm. in diameter and 3 mm. deep) of a slide was coated with the anticoagulant, dried, and an unmeasured quantity of blood, added directly from a finger puncture, was thoroughly mixed with the anticoagulant. A 2 per cent solution of a mixture of ammonium and potassium oxalate (0.8 gm. potassium oxalate and 1.2 gm. ammonium oxalate in 100 c.c. of distilled water) recommended by Kato<sup>10</sup> and Heller and Paul<sup>14</sup> was used. Like Andresen,<sup>12</sup> we found this mixture to be unreliable, even when centrifuging of the blood sample was carried out immediately. Either coagulation was not completely prevented or shrinkage occurred. Changing the concentration of the mixture and the amount placed on the depression slide, before drying and before the blood was added, did not correct the condition to the extent that the method could be considered reliable. Sodium oxalate, potassium oxalate, and sodium citrate were tried in the usually recommended concentrations, as well as in various modified concentrations, but with no better success than with the oxalate mixtures. Though fairly satisfactory packing of the red blood cells could be obtained if centrifuging was carried out immediately, satisfactory packing with delayed centrifuging could be secured only when shrinkage occurred. Since the quantity of blood added to the depression slide was not constant, even though the depression appeared filled, it was impossible to secure a precise proportion of the anticoagulants to blood. Hence shrinkage of different degrees for different samplings of blood occurred, and corrections for shrinkage could not be made

with certainty. Obviously this method of mixing inorganic anticoagulants and blood for cell volume studies lacks necessary accuracy.

A modification of the depression slide method by placing definite quantities of anticoagulant and of blood in the depression was more successful than the method already described but did not always yield accurate results. Also the method involving the use of definite quantities of blood and anticoagulant became very complicated as to technique and apparatus required for maintaining accuracy. Since, as Andresen<sup>12</sup> also has pointed out, precise methods employing inorganic anticoagulants could not always be relied upon for consistently accurate results, heparin was used as the anticoagulant. It was used as a 1 per cent solution as recommended by Rosahn,<sup>4</sup> Andresen,<sup>12</sup> and others. A drop of the solution was placed in the depression of the slide, dried, and blood added directly from a finger puncture until the depression was three-fourths to practically completely filled. This was found to prevent coagulation for long periods of time. Pipettes filled with blood from the depression slide immediately after mixing with the dried heparin showed complete and accurate packing of the blood cells after standing for 1½ hours before being centrifuged, as shown in Table I. Though good packing of the red

TABLE I  
COMPARATIVE CELL VOLUME DETERMINATIONS

SUBJECT	CELL VOLUME IN PER CENT OF HEPARINIZED CAPILLARY BLOOD COLLECTED IN DEPRESSION SLIDE AND CENTRIFUGED IN CAPILLARY PIPETTES							CELL VOLUME IN PER CENT OF OXALATED VENOUS BLOOD CENTRIFUGED IN WINTROBE TUBES		
	PERIOD OF TIME PIPETTES ALLOWED TO STAND BEFORE CENTRIFUGING									
	NONE	15 MIN.	30 MIN.	45 MIN.	1 HOUR	1¼ HOURS	1½ HOURS			
I	40.2	40.1	39.9	40.7	40.7	40.2		40.5		
II	48.4	47.3						47.0	47.0	
	47.2	47.3	46.0	46.1	46.0	45.9	46.6	47.0	47.0	
III	41.2	41.8	40.7	41.3	41.0	41.2	41.1	41.0	41.0	
								41.0	41.5	

blood cells can be secured after centrifuging is delayed for a period of an hour or more, it is advisable to centrifuge as soon as possible to insure accuracy, particularly if an angle centrifuge is used, for a few cells tend to adhere to the portion of the wall of the pipettes against which the centrifugal force is directed.

Only one precaution is necessary for the last-described technique, that the proportion of heparin to blood must not be too great. If a small drop (approximately 3.0 c. mm. for these studies) of a 1 per cent solution of heparin is used, only enough to leave a visible deposit at the edge of the depression, a precise quantity of blood need not be added. If this general rule is followed, changes in volume of the cells will not occur.

#### SUMMARY

1. The capillary pipette method of determining cell volume of capillary blood drawn directly into the pipette without previous mixing with an anticoagulant is a satisfactory method if centrifuging is carried out immediately.

Wetting the pipette with anticoagulants and blowing out the excess fluid aids in securing consistently accurate results. Inorganic anticoagulants used in concentrations ordinarily mixed with blood are, as a rule, not an aid to accuracy, stronger solutions being required. A one-third saturated sodium citrate solution is constantly effective when centrifuging is not delayed. A 1 per cent solution of heparin is also effective when used in a similar manner.

2. Mixing of dried inorganic anticoagulants and small but unmeasured quantities of capillary blood in depression slides before filling the pipettes for delayed centrifuging is subject to too great an error for practical use.

3. Only heparin thoroughly mixed with small and approximate quantities of capillary blood before the pipettes are filled permits delayed centrifuging of the blood sample with complete and accurate packing of the blood cells.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**PHOSPHATASE in Chronic Arthritis**, Steinberg, C. L., and Suter, L. C. Arch. Int. Med. 64: 483, 1939.

Determinations of the phosphatase content of the serum were made for 44 patients with atrophic arthritis, 8 patients with hypertrophic arthritis, 5 patients with mixed atrophic and hypertrophic arthritis, and 5 patients with osteitis deformans. This same study was made of 6 healthy persons. In the only case of arthritis in which the value for serum phosphatase was above 4.2 units, it was later proved that malignant tumor of the prostate gland was present, with secondary involvement of bone and lung tissue. An increase of serum phosphatase in this type of malignant disease has been described by Gutman and his co-workers. Determination of the phosphatase content of the serum in cases of chronic atrophic and hypertrophic arthritis is, therefore, important in the differential diagnosis. A normal value for serum phosphatase is characteristic of chronic atrophic or hypertrophic arthritis; an abnormal value suggests the possibility of a complicating condition or an erroneous diagnosis of this condition. Determinations of the phosphatase content of the serum should be made a routine procedure in the study of diseases of the bones and joints.

**ENDOCARDITIS**, *Streptococcus Viridans*, Middleton, W. S., and Burke, M. Am. J. M. Sc. 198: 301, 1939.

Certain details in this clinicopathologic analysis of 88 patients with *Streptococcus viridans* endocarditis lenta justify especial emphasis:

1. Further evidence is adduced to support the thesis of a close relationship between congenital and rheumatic lesions of the heart and endocarditis lenta.

2. Acute upper respiratory infections, rheumatic fever, infected abortion, dental extraction, and massage for nonspecific prostatitis, apparently served as precipitating factors in the development of certain cases of this condition.

3. Contrary to the accepted opinion, congestive heart failure may attend or mask this condition.

4. The clinical manifestations and course of this affection are notoriously varied and inconstant. After the cardiac changes incident thereto, particular attention has been directed to its toxic and its embolic features. Splenic and renal changes, including embolism, were very frequent. Mycotic aneurysms offered serious diagnostic problems. Cerebral accidents were not infrequent. Occasionally a mycotic aneurysm of a cerebral vessel may explain certain neurologic phenomena of this condition. Again the clinical picture may suggest thyrotoxicosis, and the unexplained elevation of the basal metabolic rate may add to the diagnostic confusion.

5. This study offers material support to the importance of the diagnostic triad, i.e., petechiae, splenomegaly, and a positive blood culture for the *Streptococcus viridans*. Given the background of a congenital or a rheumatic heart lesion and a remittent fever, this triad offers the logical direction of attack.

6. The prognosis of *Streptococcus viridans* endocarditis lenta is very grave. Only inferential evidence of the pace of the decline is offered by the circulatory, renal, embolic, toxic, constitutional, and hematologic reactions. Although remissions of varying durations and degrees are the rule, certain patients undergo a rapidly progressive decline. Attention has been directed to the ominous significance of the euphoria that attends late remissions.

7. This group included one instance of healed endocarditis lenta. The clinical activity apparently occurred at a period removed from the hospitalization. All therapy in the remaining number (87) was unavailing.

**PNEUMONIA, Treatment of, With Type-Specific Immune Rabbit Serum, Callomon, V. B.** Am. J. M. Sc. 198: 349, 1939.

A report is made on the treatment of 45 cases of pneumococcus pneumonia with homologous type-specific immune rabbit serum. Two deaths occurred in this series.

Premature recovery by crisis or rapid lysis ensued in 36 cases.

No serious serum reactions were observed.

**BLOOD PLATELETS, in Pernicious Anemia, Paddock, F. K., and Smith, K. E.** Am. J. M. Sc. 198: 372, 1939.

In marked pernicious anemia a definite thrombocytopenia is rarely lacking. This is borne out not only by the authors' figures of 90 counts, but also by those collected from the literature.

The platelets increase in number parallel to the red blood cell count on the institution of specific therapy.

This diminution of platelets is apparently of no value in differentiating this type of anemia from others with a macrocytosis.

**PNEUMONIA, Prognostic Significance of Eosinophiles in, Bracken, M. M.** Am. J. M. Sc. 198: 386, 1939.

Eosinophiles are entirely absent from the blood early in the disease in severe pneumonia, but may be present early in milder cases.

The appearance of eosinophiles in the blood of pneumonia patients treated with hydroxyethylapocupreine or not specifically treated is an index of recovery, and may precede clinical improvement by one to several days.

Eosinophiles were not found in the blood of patients dying of uncomplicated pneumonia.

The reappearance of eosinophiles during the course of pneumonia, while in itself a favorable sign, does not exclude the possibility of a serious and even fatal complication developing later.

**MONONUCLEOSIS, Infectious, Mitotic Leukoblasts in the Peripheral Blood in, Bowcock, H.** Am. J. M. Sc. 198: 284, 1939.

In infectious mononucleosis, mitotic leukoblasts and other unusual cell forms may appear in the peripheral blood near the peak rise of the leucocyte count. The occurrence of such immature and unusual cell forms is emphasized in order that confusion may be avoided in the diagnosis of this relatively benign illness which often resembles acute leucemia in many respects.

**CANCER, Temperature Factors in, and Embryonal Cell Growth, Smith, L. W., and Fay, T.** J. A. M. A. 113: 653, 1939.

Relatively low body surface temperatures normally exist in the segments concerned with the extremities (from 88° to 90° F.) and the breast segment (fifth thoracic) tends to maintain a higher surface temperature than the adjacent segments of the trunk. There is relative infrequency of primary or metastatic carcinoma developing in those parts of the body enjoying reduced temperatures, as compared to the organs and portions of the body where optimal high temperatures are found.

Certain clinical, pathologic, and biologic evidence indicates that young, undifferentiated cell growth and activity require an optimal temperature and that "critical" temperature levels exist below which these cells become inactive or undergo degenerative changes.

Local and general measures of "refrigeration" applied to patients suffering from hopeless metastatic carcinoma have been discussed, and the effects of these reduced temperatures on cell growth and activity, shown by serial biopsy and tissue cultures, have been indicated.

A "critical" level of around 95° F. has been noted below which undifferentiated cell growth, as exemplified by carcinoma pathologically and by the development of chick embryos normally, is arrested. Marked degenerative changes have been noted after seventy-two hours in biopsy specimens from carcinomatous tissues when continuously subjected to temperatures of 90° F. or below. Our observations have shown that normal cellular tissue is capable of withstanding 40° F. for prolonged periods of time without evidence of degenerative changes and normal reparative processes occur in carcinomatous areas maintained at this level. Tissue culture studies have confirmed these clinical observations.

Relief of pain encountered in terminal states of metastatic carcinoma has promptly followed "refrigeration" of the area involved or induced states of "hibernation" in which the patient has been maintained at levels of rectal temperature between 81° and 90° F. for periods as long as from one to five days.

Our observations suggest that the application of subcritical temperatures, through methods of local and general "refrigeration" may offer a valuable therapeutic adjunct to our present method of treatment of undifferentiated cell growth, particularly of carcinoma. Its practical clinical value and possibilities must await wider experience and more extensive and intensive observations.

#### **HORMONES, Female, Summary of Modern Views on, and Their Scope in Therapy, Cunningham, A. J. M. J. Australia 2: 58, 1939.**

A few brief facts showing the importance of the recognition of vitamin deficiency in pregnancy are the following:

1. Deficiency of vitamin A produces, among other things, alterations in the visual purple, giving rise to one of the earliest and most frequent of its symptoms, so-called night blindness or nyctalopia.

2. A not uncommon and distressing feature of pregnancy is polyneuritis; this in many cases is due to a vitamin B<sub>1</sub> deficiency.

3. Deficiency of vitamin C may give rise to dental caries of pregnancy and bleeding gums, and possibly also to disorders of the gastrointestinal function.

4. Deficiency of vitamin D tends to produce latent rickets in the fetus and also tetany in the mother.

5. The relationship of vitamin E deficiency and habitual abortion has recently been established.

These few brief facts show the interesting possibilities of vitamin therapy in pregnancy. The future will undoubtedly see further advances in vitamin research.

#### **GRANULOCYTOPENIA, in Sulfapyridine Therapy, Dolgopol, V. B., and Hobart, H. M. J. A. M. A. 113: 11, 1939.**

A review of the recent literature shows that 3 persons with granulocytopenia and a number of persons with leucopenia were observed in the course of sulfapyridine therapy.

Two new cases of granulocytopenia and 2 cases of leucopenia are reported in children with pertussis and pneumonia treated with sulfapyridine.

In the fatal case of granulocytopenia reported here, the patient lived nine days following a complete disappearance of polymorphonuclear cells. Minimal tonsillar lesions appeared two days before the patient died of lobar pneumonia. The pneumonic exudate was free of polymorphonuclear cells. The bone marrow was immature. Aplastic anemia developed in the course of granulocytopenia, but the megakaryocytes remained intact.

In the second case of granulocytopenia immature cells of the myeloid group appeared in the circulating blood with the beginning of recovery and were gradually replaced by more mature forms.

All 5 patients with granulocytopenia received large total amounts of sulfapyridine during the course of treatment.

In at least 2 cases of granulocytopenia sulfapyridine was apparently the only cause of the condition. In the other 3 cases a coincident damage to the bone marrow by the original infection or by a previous infection may be considered to be a contributory factor in the development of granulocytopenia.

In 2 cases granulocytopenia developed after the discontinuance of the sulfapyridine therapy.

A high total intake of sulfapyridine may cause granulocytopenia.

The damage to the bone marrow consists in depression of maturation of myeloid cells. The erythropoiesis may also be occasionally disturbed.

Blood counts should be made twice a week during sulfapyridine therapy and continued for two weeks after the withdrawal of the drug.

**LEUCOCYTES, Total Differential and Absolute Counts and Sedimentation Rates in Healthy Children 4-7 Years of Age, Osgood, E. E., Baker, R. L., Brownlee, I. E., Osgood, M. W., Ellis, D. M., and Cohen, W. Am. J. Dis. Child. 58: 61, 1939.**

There are no significant age or sex differences in the total, differential, or absolute leucocyte counts, or in the sedimentation rates for children 4 to 7 years of age.

**STATISTICAL AND SMOOTHED MEANS AND RANGES FOR TOTAL, DIFFERENTIAL, AND ABSOLUTE LEUCOCYTE COUNTS AND SEDIMENTATION RATES FOR CHILDREN 4 TO 7 YEARS OF AGE**

		STATISTICAL MEAN		PROBABLE ERROR DISTRIBUTION		SMOOTHED MEAN	95% RANGE
Leucocytes	per c.mm.	10,419	176	1,632	84	10,400	5,500-15,500
Neutrophiles	per cent	37.3	0.9	7.9	0.4	38*	16-60
Lobocytes	per c.mm.	2,959	124	Skew		4,000	1,500-7,500
Neutrophiles	per cent	-----	-----	-----		3*	0-10*
Rhabdocytes	per c.mm.	-----	-----	-----		250*	0-1,000*
Eosinophiles	per cent	-----	-----	-----		3*	0-8*
Lobocytes	per c.mm.	-----	-----	-----		300	0-800
Basophiles	per cent	-----	-----	-----		0.5*	0-2*
Lobocytes	per c.mm.	-----	-----	-----		50†	0-200†
Lymphocytes	per cent	48.9	1.0	10.1	0.5	48*	20-70*
	per c.mm.	5,020	140	Skew		5,000	1,500-8,500
Monocytes	per cent	-----	-----	-----		3*	0-7*
	per c.mm.	-----	-----	-----		300†	0-800†
Disintegrated cells	per cent	-----	-----	-----		5*	0-12*
	per c.mm.	-----	-----	-----		400*	0-1,200*
Sedimentation rate	15 min.	-----	-----	Skew		2†	0.5-5.0†
	45 min.	-----	-----	Skew		10†	1.0-30.0†

\*Applies also to 8- to 14-year-old boys and girls.

†Applies also to persons 8 years of age or older.

**TRANSFUSIONS, Blood, The Use of Heparin in, Sappington, S. W. J. A. M. A. 113: 22, 1939.**

A stable, potent, pure heparin was used as the anticoagulant in 40 blood transfusions and appeared to be without toxic or other undesirable effects on donor or patient. It was used in two ways: first, in vitro to heparinize the drawn blood from the donor; second, in vivo to heparinize the donor by intravenous injection. The first corresponds to transfusion of citrated blood and differs from it only in the anticoagulant. The second method is simpler in that the heparinized blood is withdrawn from the donor and introduced into the patient without any treatment outside the body. Seventeen patients were given transfusions by the first method, and 23 by the second, all with 500 c.c., since a larger amount of blood was thought a better test of the procedure. These were all done by a gravity method paralleling the citrate technique in order that comparison of the two anticoagulants could better be made.

All 40 transfusions were successful in that the entire amount of blood was transfused and the donors and patients suffered no ill effects. But it was found that for a successful

uninterrupted transfusion a larger needle was necessary for introduction into the patient than in the citrate method, a 16 gauge needle as compared with an 18 gauge commonly used in citrate transfusion. If a 500 c.c. transfusion is desirable, this has a disadvantage in that the patient's veins may be difficult to enter with such a large needle. If a small transfusion is sufficient, up to 350 or even 400 c.c. may be given without difficulty with an 18 gauge needle. Or the operation of giving 500 c.c. can be accomplished by giving as much blood as will flow through the small needle and then completing the transfusion by making another venipuncture with a fresh 18 gauge needle. The two punctures cause little or no discomfort to the patient. This hindrance to the heparin transfusion is caused by clotting within the smaller needle, even though the same heparinized blood may be kept days without clotting. In one instance blood was banked twenty-four hours and in three instances forty-eight hours and then transfused successfully. Reactions are negligible, as they are now in transfusions with citrated blood. Whether the heparin method can displace the well established citrate method remains to be seen. It would seem to offer two advantages. The heparinization of the donor makes the procedure more simple and almost a one-man job. The therapeutic value of heparin may be of advantage in post-operative transfusions.

**DIPHTHERIA, Potassium Tellurite in the Diagnosis of, Tomlin, E.** Brit. M. J., June 24, 1939, p. 1273.

The technique of this suggested clinical test for diphtheria is simple.

A negative result is of value in that it supposes with great accuracy (100 per cent in this series, 92 per cent in that of Dr. Manzullo) that the disease is not diphtheria.

It is unlikely that any case of diphtheria would be missed through reliance being placed on the test, if the technique were not at fault.

Such a high percentage of false positive results may be obtained that no definite diagnosis of diphtheria should be made on a positive result.

It is possible theoretically, and in view of the discrepancy between these results and those of Manzullo, that the proportion of false positive results will vary from place to place and from time to time.

This test can in no way take the place of the clinical and bacteriologic methods of diagnosis already in use.

**TUBERCLE BACILLI, Preservation of, Cohn, M. L.** Am. Rev. Tuberc. 40: 99, 1939.

Desiccated human (virulent and avirulent), bovine (virulent), and avian (virulent) tubercle bacilli almost completely retain viability for three years at refrigerator temperature, while natural cultures survive under the same condition of temperature only about six months to one year (occasionally two years). The loss of viability of the desiccated cultures of these same strains of tubercle bacilli is much more rapid at incubator temperature, being almost complete at six months to one year; while at room temperature, they survive a little better than at incubator temperature.

The loss of viability of tubercle bacilli is primarily a function of the temperature at which they are stored, regardless of whether they are desiccated or natural. The rate of loss of viability of cultures of tubercle bacilli is greater in the presence of oxygen, less in the presence of air, and least in nitrogen. Desiccation, although a minor factor, aids in maintaining the viability of tubercle bacilli. Desiccation can remove as much as 70 to 75 per cent of the water (and volatile materials) from the cultures of tubercle bacilli without appreciable detrimental effect on the viability. Aside from the preservation of well-grown broth cultures of mammalian tubercle bacilli (some of the bacilli remaining viable for over twelve years, as described by us in 1933), cultures of strains of mammalian and avian tubercle bacilli can be preserved in viable form for over three years when desiccated and sealed in air or nitrogen and maintained at refrigerator temperature. The latter method possesses advantages over the former in that the specimens occupy little space and are readily transportable.

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## CLINICAL AND EXPERIMENTAL

### ACTION OF OUABAIN ON THE SPLANCHNIC CIRCULATION IN THE DOG\*

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WHILE it is generally agreed that the administration of digitalis in the normal dog results in decreased cardiac output,<sup>1-6</sup> there is no agreement as to the underlying mechanism. Cohn and Stewart<sup>1, 4, 7-10</sup> attribute the decreased output to a diminution in the heart's size, while Dock and Tainter<sup>5, 6</sup> and Katz and others<sup>11</sup> believe that the predominating action of digitalis is on the peripheral vessels, producing a constriction of the hepatic veins, with resultant pooling of blood in the splanchnic area. The effect of the drug on the size of the heart has been recently discussed by Stewart<sup>10</sup> and, on the other hand, the question of diminished venous return to the heart by Katz.<sup>12</sup> At present it is impossible to say definitely whether the primary cause of the decrease in cardiac output following digitalis is due to its action on the heart or on the peripheral structures.

It has been shown in man<sup>13-15</sup> that digitalis decreases the circulating blood volume. Since such results seemed to suggest Dock and Tainter's<sup>5, 6</sup> idea that there is a pooling of blood in some of the viscera after digitalis administration, we undertook to ascertain the peripheral action of digitalis with special reference to a possible pooling of blood in the splanchnic area.

Although extensive work has been done on the action of digitalis glucosides on the isolated heart<sup>16</sup> and the peripheral vessels separately, a correlation

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of the actions of digitalis simultaneously on the heart and the peripheral vessels by visualization in the intact animal has not been made before. By such an experimental approach we hoped to clear up some of the present confusion in the previously reported results. *It is worthy of emphasis that the action of a drug, such as digitalis, on the organism as a whole is dependent not only on its action on individual organs or on some particular function but also on many interdependent readjustments which occur in the normal intact organism.*

#### METHODS

The subjects of these experiments were 42 normal, intact dogs, usually between 6 and 12.5 kg. in weight. Light anesthesia was induced by inhalation of chloroform in order to keep the animal quiet while chloralose (80 to 90 mg. per kg.) was administered intravenously. The mean arterial blood pressure was recorded with a mercury manometer joined to a cannula in the carotid artery. The respiration was recorded with a plethysmograph, and artificial respiration was used in some experiments.

The average normal animal requires a fairly fixed quantity of ouabain per unit of body weight to cause death. Nevertheless, variations are always present and occasionally may be quite marked. Likewise, the amount of ouabain required to produce a digitalis-like action on the heart is variable, but insofar as it was possible we adhered in principle to the idea of what constitutes a "therapeutic dose," as laid down by Cohn<sup>3</sup> (0.029 to 0.039 mg. per kg.). Therefore, the effect upon the heart, rather than the actual amount of the drug injected, was used as an index of whether or not a given dose could be considered comparable to a therapeutic or toxic dose. We shall intentionally refrain whenever possible from using the words "therapeutic dose" in relation to dogs, since ouabain is not used therapeutically in dogs; we have adopted the nomenclature of a "small dose" to indicate that such dosage had a definite digitalis effect on the heart but exhibited no toxicity. For a "small dose," 25 to 30 per cent of the calculated lethal dose was used. Ouabain was selected as a representative of the digitalis glucosides because its crystalline form insures uniform dosage. Further, it can be administered intravenously, and evokes a rapid response on the part of the circulation. By using this drug the effects due to saponins or other substances present in the whole leaf were eliminated.

In order to overcome various objections and possible sources of error in the work of others and to check on the validity of our own results, we studied the splanchnic vascular bed by several different methods of approach. We first investigated the volume changes of the spleen, but, because of the labile nature of the spleen and its normal rhythmical contraction and relaxation, we soon changed to the alterations of volume of intestinal loops. In the experiments on spleen and intestinal volume, oncometers were placed on the respective organs and the volume changes were recorded on the drum in the usual manner.

Because of the discrepancies which were encountered with the oncometric studies of the spleen and intestine and in order to get a clearer picture of the

action of ouabain on the blood vessels themselves, we undertook the direct visualization of the various components of the splanchnic vascular bed. This was first done by means of the abdominal endoscope designed by Marrazzi.<sup>17</sup> To increase the field under view and to make certain that the same vessels were constantly under observation during the several hours of the experiment, it became necessary to replace the endoscope with an apparatus of our own construction (see Fig. 1) and to exteriorize a loop of ileum with its mesentery. This loop was bathed continually in normal saline at body temperature. The changes in vessel caliber were established by comparing them to a hair in the field or to the markings on an ocular micrometer scale.

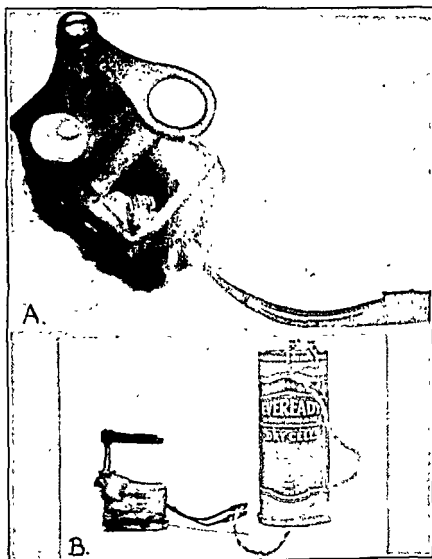


Fig. 1.—Apparatus used for visualization of vessels of the splanchnic bed. A, showing arrangement of lens and lighting system; B, showing space for mesenteric loop between lens and glass plate.

Despite the precautions taken to maintain physiologic conditions, there probably was some unavoidable effect on the mesenteric vessels incident to the operative procedures. In an attempt to control this, a period of thirty minutes to one hour was allowed to elapse before ouabain was administered. Such a pause permitted circulatory readjustments to take place and the normal variation in vessel caliber to be evaluated. This preliminary period further afforded an opportunity to observe the effects of temperature, of intravenous injection of fluid (saline), and of the action of known vasoconstrictor and vasodilator drugs (epinephrine and amyl nitrite).



## OBSERVATIONS

*Experiments on the Spleen Volume.*—The effect of intravenous injection of ouabain on the volume of the spleen was studied in 10 dogs. Following a "small dose" no appreciable change was found in 5 instances. Of the other 5 dogs, there was a diminution in the size of the spleen in 3 and an increase in 2. In the cases where a "small dose" of ouabain had no effect on the spleen volume, the amounts per kilogram of body weight varied from 0.022 to 0.06 mg. A similar variation in dosage, 0.029 to 0.056 mg. per kg., caused a decrease in the volume of the spleen. Likewise, 0.03 to 0.08 mg. per kg. caused an increase in spleen volume. In 5 cases either repeated small doses or a single large dose (0.058 to 0.123 mg. per kg.) were given to study the toxic effect of ouabain. The predominating effect (3 cases) was a constriction of the spleen, and of the other 2 animals, one exhibited dilatation and one, no change. This is shown in Table I.

TABLE I  
SHOWING THE EFFECT OF OUABAIN ON VOLUME OF SPLEEN AND INTESTINAL LOOPS

	SMALL DOSE			LARGE DOSE		
	NO CHANGE	DILATATION	CONSTRIC- TION	NO CHANGE	DILATATION	CONSTRIC- TION
Spleen	5	2	3	1	1	3
Intestine	3	5	3	0	8	0

*Experiments on the Intestinal Volume.*—Eleven animals were studied in the experiments on intestinal volume. In 5 of these the predominating effect of a "small dose" of ouabain was dilatation. In the remaining 6 dogs, there was a decrease in intestinal volume in 3, and no change in the other 3. In 8 animals in which either the first dose of ouabain proved to be toxic or the "small doses" were given repeatedly until toxicity resulted, the intestinal volume was increased in every instance (see Table I).

*Experiments on the Visualization of the Vascular Bed of an Intestinal Loop.*—We have complete data of the effects of ouabain on the vascular bed of 18 dogs. As in the case of the experiments on the volume changes of the spleen and intestine, these experiments have also been divided into two series, those dealing with the effect of a "small dose" and those with large or toxic doses.

A typical protocol is shown in Table II and a summary of all the experiments is shown in Table III. An analysis of these experiments shows that with a "small dose" of ouabain the predominating effect was no change at all, this being the result obtained in at least one-half of the dogs studied. In those animals that did exhibit a change, constriction was the outstanding feature. This constriction was most obvious in the large arteries and least prominent on the venous side of the vascular tree.

In the experiments with "small doses" of ouabain, dilatation was relatively inconspicuous. On the other hand, with large or toxic doses dilatation becomes a very conspicuous feature and in contrast to the constriction with "small doses," which was prominent on the arterial side of the vascular loop.

the dilatation was most prominent on the venous side of the loop. In a small percentage of cases dilatation was present throughout the entire vascular tree. Some constriction was also present with toxic doses but it seemed to be mainly limited to the medium and very small arterioles. These effects are graphically shown in Fig. 2.

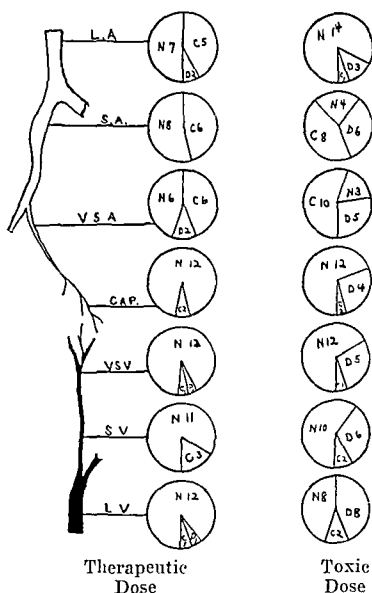


Fig. 2.—Graphic illustration of the effects of ouabain on the intestinal vascular tree with small and large doses. Abbreviations same as for Table II.

In all animal experiments dealing with the circulation the question as to how great a factor alterations in oxygenation may play in the results obtained was seriously considered. While there is no doubt that moderate asphyxia or hyperventilation could produce changes in the blood flow through the splanchnic area, every attempt was made to reduce this variable to a minimum. In some experiments artificial respiration was used throughout, and in comparing the results in these instances with those in which artificial respiration was not employed, no difference in result was apparent. In certain cases where the respiration was slow, artificial respiration was instituted to insure adequate ventilation; however, no appreciable changes were noted in either direction from the trends obtained. We feel, therefore, that the variations described were not due to changes in oxygenation of the blood. In an attempt to explain the results on the basis of other experimental variables we analyzed our work in relation to the dosage of ouabain, blood pressure, and heart rate.

TABLE II  
SHOWING THE EFFECT OF OUABAIN ON THE VASCULAR BED OF THE MESENTERY  
Experiment No. 101  
Dog—9 kg.

TIME	COMMENTS	P.	B. P.	EFFECT ON VASCULAR BED						
				L. A.	S. A.	V. S. A.	CAP.	V. S. V.	S. V.	L. V.
10:00	CHCl <sub>3</sub>									
10:05	Chloralosane									
10:10	Art. resp.									
10:15										
10:41	Ouabain 0.25 mg.	90	140	*						
10:42		90	140	N	N	N	N	N	N	N
10:43		90	145	N	N	N	N	N	N	N
10:44				N	C	C	N	N	N	N
10:45		90	142	N	C	C	N	N	N	N
10:46½	Ouabain 0.25 mg.	90	140	N	N	N	N	2D	D	N
10:47				N	N	D	N	2D	N	N
10:48		90	140	N	N	N	N	2D	N	N
10:53			140	N	N	N	N	2D	N	N
10:55		95	140	N	N	N	N	N	N	N
10:56		95	140	N	N	N	N	N	N	N
10:58		100	146	N	N	N	N	N	N	N
10:58½		100	146	N	N	N	N	N	N	N
10:59				N	D	D	N	N	N	N
10:59½		90	150	N	N	N	N	N	N	N
11:00	Ouabain 0.25 mg.		150	N	N	N	N	N	N	N
11:01		90	152	N	N	N	N	2D	2D	D
11:03		90	152	N	N	N	N	D	D	D
11:08		90	152	N	N	N	N	D	D	D
11:12		90	156	N	N	N	N	D	D	D
11:12½		90	156	N	N	N	N	D	D	D
11:13½		100		N	N	N	N	D	D	D
11:15½		210	200	N	N	N	N	N	N	N
11:17		200	208	N	2C	2C	D	2D	3D	2D
11:19		200	210	2D	2D	2D	2D	2D	3D	2D
11:24		110	200	2D	2D	2D	4D	4D	4D	4D
11:46		100	175	2D	2D	2D	4D	4D	4D	4D
		110	170	N	D	N	N	N	N	N

\*Preliminary period of observations. All future changes in vessels are in relation to this norm.

#### Explanation of Abbreviations

P.—Pulse	4D—Dilatation, marked
B.P.—Blood pressure	N—No appreciable change
C—Constriction, slight to 10 per cent	L.A.—Large artery
2C—Constriction, to 20 per cent	S.A.—Small artery
3C—Constriction, to 50 per cent	V.S.A.—Very small artery
4C—Constriction, marked	Cap.—Capillary
D—Dilatation, slight to 10 per cent	V.S.V.—Very small vein
2D—Dilatation, to 20 per cent	S.V.—Small vein
3D—Dilatation, to 50 per cent	L.V.—Large vein

An analysis of the dose of ouabain and the effect on the spleen and intestinal volume showed no relationship, except insofar as the results with "small doses" varied from those with large doses. In other words, the variations within a certain group of experiments with either "small" or toxic doses of ouabain cannot be explained on the basis of dosage.

Ouabain in "small doses," as has been repeatedly demonstrated by others, usually caused a rise in arterial pressure. When toxic doses were used, the arterial pressure fell at first slowly but terminally, rather abruptly. There was no particular correlation in the results obtained with respect to the initial pressure. Those experiments with an initial low arterial pressure were similar to those with an elevated arterial pressure. Furthermore, the degree of change

TABLE III

SUMMARY OF THE EFFECT OF OUABAIN ON THE VASCULAR BED OF THE MESENTERY

AT TIME OUABAIN INJECTION WAS BEGUN			CHANGES FOLLOWING OUABAIN EFFECT ON SPLANCHNIC BED								
EXP.	B. P.	P.	B. P.	P.	L. A.	S. A.	V. S. A.	CAP.	V. S. V.	S. V.	L. V.
CHANGES FOLLOWING OUABAIN IN SMALL DOSES											
819	155	100	210	60	2D	2C	1D	1C	N	N	N
823	110	75	91	60	3C	3C	3C	3C	3C	3C	3C
824	135	150	154	155	N	N	N	N	N	C	N
831	100	100	120	100	2C	3C	2C	N	N	N	N
92	70	90	90	75	2C	3C	3C	?	N	N	D
910	165	60	195	120	C	C	N	N	N	C	N
920	150	85	170	80	C	C	N	N	N	N	N
921	225	70	250	60	D	N	C	N	N	N	N
930	180	100	195	75	N	N	C	N	N	N	N
101	140	90	142	90	N	N	D	N	2D	N	N
105	156	180	177	140	N	N	N	N	N	N	N
113	118	160	139	155	N	N	C	N	N	N	N
115	110	95	60	100	N	N	N	N	N	N	N
119	175	150	184	130	N	N	N	N	N	N	N
CHANGES FOLLOWING OUABAIN IN TOXIC DOSES											
823	91	60	112	60	D	D	2D	4D	3D	2D	2D
824	154	90	156	60	N	4C	3C	4D	2D	N	N
910	170	150	202	115	N	C	2C	N	N	N	N
920	180	250	144	225	2C	2C	3C	4C	1D	2D	D
921	250	45	215	50	N	3C	3C	N	N	N	N
930	180	165	255	50	N	C	2C	N	N	N	D
101	156	100	210	200	D	D	D	3D	4D	4D	4D
105	160	95	200	80	N	D	D	N	N	N	N
113	140	120	160	100	N	N	N	N	N	N	N
119	165	120	195	100	N	N	N	N	N	N	N
914	180	200	190	140	N	N	N	N	N	N	N
918	160	90	200	84	N	N	C	N	N	N	N
102	80	90	110	75	N	C	2C	2C	2C	C	C
93	225	70	250	60	N	D	2D	3D	2D	2D	2D
98	114	78	84	40	N	C	C	N	N	C	C
108	150	110	210	80	D	C	C	N	N	N	D
820	240	130	215	90	N	D	D	N	N	D	D
114	220	120	220	98	N	D	C	N	N	D	D

For explanation of abbreviations see Table II.

in pressure following the administration of ouabain lacked any definite relationship with the changes in the splanchnic vascular bed. Likewise, an analysis of the heart rate in the various instances failed to explain the results obtained. Because of this we believe that the results described must be due to the action of the drug itself on the vessels of the splanchnic bed.

## DISCUSSION

We have demonstrated that following the administration of ouabain to normal dogs, there was a distinct difference in the action on the splanchnic bed of a "small dose," comparable to that of a therapeutic dose in man and a large or toxic dose. The effect of a "small dose" on the volume of the spleen is variable. While in some instances dilatation of the spleen was noted, as was found by Dock and Tainter,<sup>5</sup> in an equal number of cases constriction occurred, and in the majority of instances there was no appreciable effect. The conclusions reached by Dock and Tainter on the basis of experiments on two animals seems unjustifiable, for as our results suggest, if they had employed a larger number of animals, their results might be less uniform. The changes found

in the spleen do not appear to us to be due to the action of ouabain but rather to other variables in the regulation of the circulating blood volume.

With these "small doses" of ouabain increase in the intestinal volume was present in the majority of cases. Thus, with no significant change in the spleen volume and an increase in volume in the mesenteric bed, we may conclude that there is some pooling of blood in the splanchnic area following the administration of these "small doses." More detailed examination of the mesenteric tree in similar cases shows that this pooling took place by dilatation of the large and medium-sized arteries and veins. Because the observed increase in the intestinal volume was not very great and the dilatation of the vessels, by actual visualization, was rather uncommon, and further because the large vessels are only a small part of the mesenteric bed, we feel that the amount of pooling in these instances could not have been very great.

With toxic doses of ouabain there was a striking decrease in the size of the spleen. This effect is well known, having been demonstrated repeatedly.<sup>13</sup> On the other hand, such doses caused the volume of the intestinal mesentery to increase, i.e., the vessels dilated, in every instance. Careful study of the various components of the vascular tree shows that the bulk of the blood is retained on the venous side. In view of the fact that the mesenteric bed is of greater volume than the spleen, one may assume that with toxic doses there is a definite pooling of blood in the splanchnic area, even though the spleen volume was diminished.

The volume of blood that is pooled in the mesenteric bed is believed by Dock and Tainter<sup>5, 6</sup> to be caused by constriction of the hepatic veins. Although we have no direct observations as to whether or not this accumulation of blood in the splanchnic area is the result of an obstructed hepatic outflow (hepatic vein constriction), still obstruction of hepatic flow should always cause increase in spleen volume; this was certainly not present. Furthermore, on the basis of our detailed study of the various units of the vascular tree, where in some instances part of the venous tree dilated while other areas remained unchanged (see Table III), it appears that this dilatation is not a purely passive mechanism. While ouabain undoubtedly does affect the vascular tree, causing some pooling of blood in the mesenteric bed, more so with large doses than with "small" ones, our evidence does not permit us to go to the extreme that Dock and Tainter, and Katz, Rodbard, Friend, and Rotterman<sup>11</sup> have gone when they conclude that the "site of action of therapeutic doses of digitalis is on the peripheral vessels" and on the heart indirectly. This explanation of the mechanism of diminished cardiac outflow seems unlikely for other reasons as well. The effect of the action of ouabain upon the heart endures for a much longer period than on the splanchnic vessels. As pointed out by Cohn and Steele:<sup>10</sup> "If, as between effect on heart and on hepatic veins, that on veins persists, it is essential to know in general how long this action may be. That on the heart is known to last for several perhaps for many days. If that on the veins is as long, the action of both structures must contribute to the final result; if not, that on the veins possesses a slight influence only." The evidence we have presented shows that

the effect on the veins is of much shorter duration than on the heart. Furthermore, as has been pointed out, with "small doses" of ouabain the amount of pooling of blood is rather small and does not seem sufficient to account for the decrease in cardiac output.

#### SUMMARY

1. The action of ouabain on the splanchnic circulation was investigated by several methods in 42 intact dogs.

2. "Small doses" of ouabain, comparable to a therapeutic dose in man, had no significant effect on the spleen volume; large or toxic doses caused a decrease in spleen volume.

3. The volume of intestinal loops increased slightly with "small doses" of ouabain and showed a striking increase with toxic doses.

4. By making the vascular tree of the mesenteric bed visible the site of action of the drug was demonstrated.

5. Our results indicate that there is some pooling of blood in the splanchnic circulation following "small doses" but that the amount is rather small and does not seem sufficient to account for the decrease in cardiac output. Furthermore, this pooling of blood is not a purely passive affair, as one might expect if it were due to constriction of the hepatic veins.

It is a pleasure to acknowledge the helpful suggestions given by Dr. Arthur C. DeGraff and Dr. George B. Wallace.

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## ORAL POLLEN THERAPY\*

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ALTHOUGH there have appeared a number of reports indicating that in pollen-sensitive individuals desensitization can be produced by the oral administration of pollen antigens, this method of treatment in hay fever is considered by the majority of allergists as yet in the experimental stage.

The early experiments of Curtis,<sup>1</sup> Touart,<sup>2</sup> Walzer,<sup>3</sup> and Thommen<sup>4</sup> indicated that in certain cases of hay fever satisfactory relief of symptoms could be obtained by some form of specific oral pollen therapy. No extensive application of this method was attempted until Black,<sup>5, 6</sup> after demonstrating that ragweed antigen appears in the blood and urine after ingestion of the pollen, used the oral route in the treatment of 150 grass and ragweed-sensitive patients. He used as the initial dose 10 drops of a 1:20 (5 per cent) pollen extract, increasing it rapidly to a maximum dosage of 60 drops three times daily in water, milk, or other beverage. Although having considerable success with the oral method, his results did not equal those he obtained with the method of parenteral injections.

Later, Gatterdam,<sup>7, 8</sup> using less concentrated pollen extracts and smaller doses than did Black, reported marked relief with the oral treatment in 75 to 85 per cent of hay fever patients. Black and Gatterdam pointed out several advantages of oral therapy in hay fever, such as the wide margin of safety, the ability of many patients to reach protective doses rapidly, the minimum of expense and discomfort to the patient, and its application, in selected cases, in combination with hypodermic therapy.

Stier and Hollister,<sup>9</sup> after some preliminary experiments with oral pollen therapy, observed that Black's recommended doses were too large and that, in some patients, almost unbelievably small oral doses must be given to relieve symptoms, since the larger doses would produce them. They attempted in a few patients the alternation of hypodermic doses of pollen extracts and oral doses with satisfactory results. In other patients, who desired to dispense with

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hypodermic injections while away on vacations, the substitution of oral treatment during these periods was found to be effective in maintaining their desensitization. They then used oral pollen therapy exclusively for the following three years in 383 patients, and reported that 78 per cent of these patients obtained satisfactory relief from hay fever symptoms. They believe that their results with oral therapy in hay fever compare very favorably with the results obtained with the hypodermic administration of pollen extracts.

In Europe good results with oral therapy in pollen-sensitive patients are claimed by Urbach,<sup>10</sup> who employed the appropriate pollen peptones or proteins prepared from the entire pollinating flower, grass, or grain for treatment, although using the pollens in testing for sensitivity.

Bernstein and Feinberg<sup>11</sup> treated 20 ragweed-sensitive patients in a Middle West community by the oral administration of a 1:33 (3 per cent) ragweed pollen extract. Treatment was given co-seasonally three times daily, starting with 1 drop doses and increasing to 10 to 15 drop doses of this extract at the end of one week. Although they conclude that oral ragweed pollen therapy is of little value in this region, it should be noted that their dosage was higher than used by Gatterdam or Stier and Hollister, that the treatment period was short, from one to three weeks, and that the majority of their patients had received previous hypodermic therapy without benefit.

Rockwell<sup>12</sup> used a specially prepared whole pollen antigen in capsules in the oral treatment of 182 patients with grass or ragweed sensitivity. His initial dose was 500 pollen units, and the usual maximum dose for grass-sensitive patients was 60,000 units, and for ragweed-sensitive patients, 120,000 units. Satisfactory to complete relief was obtained in 63.2 per cent of the patients, with somewhat better results in the grass-sensitive group than obtained in the ragweed-sensitive group. He believes that results comparable to those with the injection method will be obtained if oral treatment is begun preseasonally, and a sufficient maintenance dose is reached by the beginning of the hay fever season. Bohner,<sup>13</sup> using the same antigen preparation for oral administration as employed by Rockwell, reported the results of treatment in 62 ragweed-sensitive patients during a season with a very high local pollen count. Satisfactory relief was obtained in 13 out of 21 patients with oral therapy, 16 out of 21 with hypodermic injections, and 9 out of 20 with intracutaneous injections of pollen extract.

Barksdale<sup>14</sup> treated 43 patients with hay fever and bronchial asthma by the oral administration of a combination of aqueous extracts of local pollens and house dust from the patient's home. Complete or marked relief of symptoms was obtained in 88 per cent of his patients. Treatment was continued until 2 or 3 ounces of the extracts had been taken, following which, in the great majority of patients, he noted a period of desensitization lasting twelve months or more.

Black<sup>15</sup> again attempted oral therapy in 40 ragweed-sensitive patients in 1938. In this series he used pollen antigen in tablet form, starting with an initial dose of 500 pollen units, and increasing by 500 units each day until symptoms subsided, or until a dose of 4,000 units was reached. With the



larger doses, 1,000 units were taken at a time, and as many doses used during the day as was necessary to get the required amount. His doses were preferably taken on an empty stomach, followed by a glass of water. Two patients had nausea and abdominal distress, and discontinued treatment. Satisfactory results were obtained in 40 per cent of these cases. He stated that, although the results in general were not as good as with hypodermic therapy, some patients obtained complete relief of symptoms with the oral treatment.

That a certain proportion of sufferers from hay fever can be satisfactorily relieved by oral therapy seems to be established. It is not surprising that the individual reports of experiments in oral pollen therapy have been apparently conflicting, when one considers the wide divergence in the materials and methods of administration used by the various investigators. As further observations would appear to be of value, I present my experience with the oral method of desensitization in hay fever.

#### MATERIALS AND METHODS

Oral pollen therapy in hay fever was attempted in 33 patients during the latter part of 1936, and in 65 patients in 1937. The results obtained in 1936 were reported by McGrew,<sup>16</sup> who assisted in the earlier part of the experiment. Good to complete relief of symptoms was obtained in approximately 88 per cent of patients with coseasonal oral treatment. In 1937 it was decided to discontinue desensitization by hypodermic injection, and to employ the oral method for one entire season in all cases of pollen sensitivity presenting themselves for treatment at the Outpatient Clinic of William Beaumont General Hospital.

*Pollens Used.*—In 1936 there were only three pollen extracts available for oral treatment. All patients were given a combination of equal parts of pollen extracts of Bermuda grass (*Capriola dactylon*), Russian thistle (*Salsola pestifer*), and careless weed (*Amaranthus palmeri*), the three main pollen offenders in this region.\* For the 1937 season, additional extracts for oral treatment were prepared from the pollens of Western ragweed (*Ambrosia psilostachya*), sagebrush (*Artemesia tridentata*), and six tree pollens of reputed local importance. Among other pollens available only for sensitivity tests that appeared of sufficient importance to list for possible future use in this locality were the shad scale (*Atriplex canescens*) and the salt cedar (*Tamarix gallica*).

*Sensitivity Tests.*—Sensitivity tests were made by the scratch method. In the majority of patients multiple pollen sensitivity was present, usually to four or more pollens, only one patient showing sensitivity to a single pollen.

*Extract of Pollen for Oral Administration.*—The pollen extract used for oral administration was one of the extracts which have given good results in the experience of Gatterdam.<sup>17</sup> This is a 1 per cent (1:100) pollen extract in an extracting fluid consisting of 0.85 per cent of sodium chloride to which is added 5 per cent glucose and 15 per cent alcohol. The extraction is carried out at room temperature with occasional shaking for twenty-four hours, and the pollen removed by filtration through paper. This extract was used in the

\*Much of the material used to initiate the experiment was furnished by Dr. E. A. Gatterdam, of Phoenix, to whom grateful acknowledgment is given.

majority of cases. For some, the strength of the extracts used was reduced to 0.5 per cent (1:200) with satisfactory results.

*Dosage.*—The patients were instructed to start with a dose of 1 drop of the pollen extract in one-half glass of water after morning and evening meals, gradually increasing the dose by 1 or 2 drops daily until symptoms were relieved. They were instructed to watch for symptoms of an overdose, which would probably be manifested by an increase in hay fever symptoms within an hour, or a mild urticaria, or gastric distress or nausea. Gastric symptoms did not occur in this series, except in 2 patients who experienced moderate nausea at the beginning of the treatment. Many patients noted an increase in hay fever symptoms, and some experienced mild urticaria following certain doses of the extract. In such cases the patient's individual tolerance to the extract could be determined. If overdosage appeared probable, the patient was instructed to reduce the next dose by several drops, and, if symptoms were not then controlled, to increase successive doses very slowly. Often it was possible for the patient later to reach a dose equal to or considerably larger than that which previously had been above his tolerance. Some patients appeared to show a very narrow limit between the dose which controlled symptoms and that which constituted an overdose, and in some it was difficult to determine whether or not continuance of symptoms was due to overdosage or to insufficient desensitization.

The dosage which controlled symptoms in the patients treated with satisfactory results ranged from 2 drops to 17 drops of the extract twice daily, averaging from 4 to 6 drops two or three times daily (approximately 2,500 to 3,700 pollen units). In patients commencing treatment after the onset of symptoms, many were satisfactorily relieved after three or four days of oral treatment.

*Pollen Extracts Used.*—Forty-eight patients were treated with a mixture of equal parts of the extracts of the three main pollen offenders in this region; namely, Bermuda grass, careless weed, and Russian thistle. Nine patients were given one additional pollen extract, usually Western ragweed, sagebrush, or mesquite, and one patient was not completely relieved until a fifth extract was added. Three patients were satisfactorily treated with a mixture of two pollen extracts. In only 3 patients was a single pollen extract used; mesquite in one and ragweed in 2.

Patients sensitive to giant and short ragweed have shown sensitivity to the Western ragweed, but in this locality the ragweed is not of major importance and most of these patients do not require treatment for this sensitivity. One patient, an army officer, sensitive to Russian thistle, pigweeds, and ragweeds, was unable to take hypodermic treatment while on maneuvers in central Texas, where the Eastern ragweed varieties are present. This patient was satisfactorily treated by oral administration of three pollen extracts representing the sensitivity groups, in which the ragweed fraction was prepared from the Western ragweed species. Another ragweed-sensitive patient, also an army officer, was on temporary duty in Ohio during the ragweed season. After severe hay fever symptoms and asthma had appeared, Western ragweed extract was

sent to him by mail. He was relieved by oral treatment in a few days, and continued through the season without symptoms on a dosage of 6 drops three times daily. A third ragweed-sensitive patient, living in North Carolina, was furnished Western ragweed extract for oral treatment three weeks prior to the ragweed season. He took 20 drops of the extract daily until the beginning of the season, then reduced to 6 drops twice daily, with complete relief from his usual severe symptoms.

### RESULTS

Of the 65 patients treated by the oral method in 1937, 26 received complete relief from symptoms and 31 had satisfactory relief, the latter group having occasional symptoms during the dust storms common to this region, or when under other excessive pollen exposure. Considering the usual definition of satisfactory treatment in hay fever, 87 per cent of the patients were satisfactorily relieved of symptoms with oral therapy, which results approximate closely those which can be expected with the injection method of treatment.

The results in the remaining 8 patients were not satisfactory, although all but one received partial relief with the oral treatment. In at least 3 of these patients, symptoms were possibly due to sensitivity to allergens other than pollens. In one, added sensitivity to various flowers appeared probable, for which no treatment was available. In 2 patients overdosing was believed to have been responsible for the unsatisfactory results. In one patient with somewhat doubtful symptoms of pollen sensitivity, oral therapy was a complete failure, as had been previous treatment by the hypodermic method.

In the entire series 49 were adults and 16 were children. As a group, the children showed less severe symptoms and somewhat more satisfactory results with oral therapy than did the adults. Eighteen patients had taken oral treatment the previous year, and had as good or better results during the second year of oral treatment. Sixteen patients had received treatment by the injection method in previous years. Nine of these stated that the results of oral therapy equaled those received with the hypodermic injections, and 6 claimed that the oral method was superior. The success with oral therapy in these patients is believed to be due in part, at least, to the inclusion of a greater number of pollen extracts in oral treatment than had been employed in the hypodermic method.

There were 13 patients who were known to be sensitive to foods or miscellaneous allergens in addition to pollens. No specific treatment, except for pollen sensitivity, was employed, the other methods being dietary restriction or removal of offending substances where possible. Five of these patients had asthma which was relieved in four cases by oral pollen therapy and other recommended measures. Eight patients in whom asthma was one of the manifestations of hay fever were relieved of this symptom by oral therapy alone.

I personally observed all but 3 patients at intervals throughout the pollen seasons. Observation is considered important from the standpoint of obtaining the best results in oral treatment. Each patient presents an individual problem. Occasionally new sensitizations are discovered. In multiple sensitivities, ex-

tract mixtures have been changed or new extracts have been added as considered necessary. Such flexibility of treatment, without danger to the patient, is one of the advantages of oral therapy.

#### DISCUSSION

*Pollen Antigens.*—There is considerable variation in the types of pollen antigens which have been used in oral desensitization experiments. Whole pollen, pollen proteins, and different types of extracts of pollen have been used with more or less success. Alum-precipitated pollen extracts, used in hypodermic treatment by Sledge,<sup>18</sup> have not yet been tried in oral therapy. I have used one of the liquid extracts exclusively, with results not justifying a change to some other preparation. Further experimentation will be necessary to determine the most suitable type of pollen antigen for oral administration; however, the dosage will still remain the most important consideration.

There is accumulating evidence of immunologic relationship in the allergenic substances of the pollen of plant groups of related species. Thommen<sup>19</sup> states that the hay fever excitant is similar in character in the various species of pollen belonging to any particular genus of trees. He also demonstrated that a patient sensitive to one grass pollen reacts to all other grass pollens. Sellers<sup>20</sup> expressed the belief that Western ragweed is of atopic identity with the giant and short varieties. Although it is generally believed that immunologic relationship of pollens does not extend beyond the plant families, Sellers believes that sharp specificity is lacking between related families of the order *Chenopodiales*. These facts have been utilized as an aid in the selection of fewer pollen antigens for sensitivity tests and in treatment; for example, French<sup>21</sup> and others have used timothy grass pollen exclusively for testing and treating all grass-sensitive patients, and similarly a single pollen for each of the ragweed and amaranth groups.

There is also some evidence that the allergenic substance of a pollen may be present in other parts of the plant itself. Farmer<sup>22</sup> quotes the botanist Mez as believing that "all living cells of the same plant show a similarity of their reactions"; however, he records some interesting experiments with a number of plants, showing that there is no immunologic relationship between the pollens and seeds of the same plant. Curtis utilized extracts of the entire pollinating flower, and Urbach used proteins of the appropriate grass, grain, or flowers in their experiments in oral therapy. It would be well to investigate further the possibility of substituting for pollen a more readily procurable antigen from some other part of the plant.

*Dosage.*—In reviewing experiments with oral pollen therapy, the most variable factor appears to be the dosage employed. The dosage schedule successfully used in the hypodermic method is not applicable in oral therapy. The report of Bernstein and Feinberg includes observations indicating that the amount of antigen demonstrable in the blood after oral administration is only 1/4,000 of the amount demonstrated after hypodermic injection. This has led to experiments in which oral doses considerably in excess of the usual hypodermic doses have been administered; however, judging from some of the reports and from our own experience there seems to occur a far greater desensi-

tizing effect after oral administration of moderate or small doses of pollen antigen than can be readily explained by the amount of antigen which is apparently recoverable from the blood following this method of administration.

Those who have reported the best results with oral therapy have used in general small doses frequently repeated. The usual maintenance dose in our series has been from 4 to 6 drops of the mixed 1:100 extracts (approximately 2,500 to 3,700 total pollen units) taken two or three times daily.

It has been shown by a number of workers that when desensitization is obtained through oral therapy, a satisfactory desensitizing dose may be reached by the patient very rapidly. I have noted marked to complete relief after the fourth or fifth dose of pollen extract administered orally in gradually ascending doses. The desensitization period following an adequate oral dose appears to be very brief as compared to that following a maintenance dose by injection. This makes it necessary to repeat the minimum oral maintenance dose frequently up to three, and occasionally four, times a day.

*Preseasonal Treatment.*—In his experiments with oral therapy, Touart used small daily doses of antigen preseasonally for six weeks, discontinuing the treatment at the onset of the pollinating season. Some of his patients were satisfactorily relieved during the entire season. Other attempts to use this method have not produced satisfactory results. Preseasonal oral therapy alone has been discontinued in later experiments which favor the coseasonal method. Gatterdam recommends the building up of immunity with larger doses preseasonally, reducing the seasonal treatment to the lowest maintenance dose which will relieve symptoms. Rockwell believes that "build up" doses should be attempted before the season, and maximum doses continued coseasonally. In our series, preseasonal treatment followed by the minimum required maintenance dosage during the season has given good results, and is advised for patients who have not been entirely relieved by previous coseasonal oral treatment.

*Coseasonal Treatment.*—Coseasonal treatment has been the method most commonly employed in oral therapy. Stier and Hollister both favor it. Black has used it exclusively. In most of our patients it was used of necessity, as they were first seen after symptoms had become manifest. The rapidity and safety with which a maintenance dose can often be reached coseasonally are marked advantages in oral therapy. In most of our patients coseasonal treatment is all that has been required.

*Perennial or Annual Treatment.*—Gatterdam advocates building up to a moderate oral maintenance dose, repeating this throughout the year at from three- to seven-day intervals, thereby producing a more lasting immunity. This method appears worthy of further trial.

*Combined Oral and Hypodermic Treatment.*—Gatterdam employs oral therapy as an adjunct to hypodermic injections in patients not responding well to oral treatment alone. Stier and Hollister found that, in patients undergoing hypodermic treatment, oral therapy was successful in keeping up desensitization in patients undergoing hypodermic treatment during absence on vacations. Black reported that pollen administered orally to patients having severe reactions with hypodermic therapy and unable to reach protecting doses at the beginning of the season, enabled them to continue the hypodermic treat-

ment more rapidly and without reactions. It seems probable that combined oral and hypodermic treatment would produce better results than either method used alone in any series of cases.

#### DISADVANTAGES OF ORAL THERAPY

(a) *Cost*: That oral therapy is expensive has been noted in several reports. Pollens are now less expensive than formerly. If the 1 per cent (1:100) or weaker extracts are used for oral administration and dosage kept to the minimum requirements, the cost is not excessive. From the standpoint of the patient, the expense incurred in visits to the physician's office is reduced.

(b) *Results Not as Good as With Hypodermic Therapy*: This apparently applies to the giant and short ragweed-sensitive patients only, judging from the available reports. The type of extracts and dosages used by Gatterdam, Stier and Hollister, and myself have not been given an extensive trial in this group of cases. Stier and Hollister are of the opinion that better results might be expected in the fall hay fever cases if treatment were more inclusive. In our series we have included, as a rule, at least three pollens for the fall cases, usually including Bermuda grass, although skin sensitivity tests or symptoms did not necessarily indicate the need for grass treatment.

(c) *Dosages Not Easily Controlled*: This is a considerable disadvantage in oral therapy. Reactions to treatment cannot be observed with the exactness obtainable in hypodermic therapy. As the treatment is carried out at home, the patient may be inclined to experiment or overdose and not follow instructions. Even with cooperative patients it is occasionally difficult to determine the correct individual dose. Most of our patients have been sincerely interested in obtaining relief, and, as a rule, cooperate in following instructions. Small amounts of the extract were given out at each visit, requiring the patient to return frequently for refills. At these times progress was noted, evidence pointing to overdosage or underdosage was obtained, and further instructions and advice were given. In a number of patients these instructions regarding the regulation of the dosage were necessary. In oral therapy, as has been pointed out by others, each patient is very much an individual problem, and no exact schedule of dosage should be attempted.

(d) *Variability in Enteral Absorption*: There is evidence of variability of absorption of proteins through the gastrointestinal tract. In what way this may apply to the absorption of pollen antigens administered orally in allergic individuals is not known. The active principle in pollens is apparently not significantly altered by peptic digestion; however, some contrary observations have been made. Even the formerly accepted belief that pollen antigen is purely a protein is now a matter of some dispute. In oral pollen therapy the probability of individual variation in enteral absorption should be kept in mind.

#### ADVANTAGES OF ORAL THERAPY

(a) *Absence of Serious Reactions*: No serious reactions have been encountered in any of the reported experiments in oral therapy. Gastrointestinal symptoms, usually mild in character, are most frequently noted. These usually accompany the first few doses, particularly if taken on an empty stomach. Such

reactions appear to occur more frequently when pollen antigens are administered in tablet or capsule form. With pollen extracts taken after meals in liquid form, well diluted, gastric reactions have been negligible. Mild urticarial reactions, which I have not infrequently encountered, and the increase in hay fever symptoms following shortly after oral administration, have occurred in patients who have increased dosage too rapidly; most frequently they are an indication that the maintenance dose has been exceeded.

(b) *Ease of Administration:* The wide margin of safety in oral therapy permits the carrying out of treatment at home with occasional visits to the physician for instructions or advice when required. This feature is most acceptable to patients of all ages, especially to children or those averse to hypodermic injections.

(c) *Rapidity With Which Desensitizing Doses Are Reached:* Our experience is similar to that of others who have found that with oral therapy relief of symptoms can often be obtained after the first few doses or within one to three days. In many of our patients maintenance doses were reached within a week. Such results have been most gratifying when obtained in patients presenting themselves with well-developed hay fever symptoms.

(d) *Availability of Treatment Under Unusual Conditions:* This has been previously mentioned in connection with patients unable to come for office treatments, or those away on vacations, or those who travel frequently. In such cases, oral treatment offers the best solution, either given alone or as a supplement to, or temporary substitute for, hypodermic therapy.

#### SUMMARY AND CONCLUSIONS

Oral pollen therapy has been employed at William Beaumont General Hospital, El Paso, Texas, in the treatment of patients with hay fever over a period of three years. In 154 observed cases the results have been comparable to those which would have been expected if the hypodermic method of treatment had been used. All patients except 3 came from the semiarid region of west Texas. These 3 were ragweed-sensitive patients who were given oral treatment, using a Western ragweed extract, when under exposure to the Eastern species, and were completely relieved of symptoms.

Materials and methods used are set forth and discussed, together with the reports of other investigators in oral pollen therapy.

In the present-day treatment of hay fever, desensitization by the oral administration of specific pollen antigens cannot entirely supplant the established method of parenteral injections. However, a considerable number of patients can be given as much relief by intelligently administered oral therapy as can be obtained by the hypodermic method. Under certain circumstances treatment by the oral route is the method of choice. There is sufficient evidence regarding the efficacy of oral therapy in hay fever to justify a more extended use of this method.

*Addendum.*—During 1938 oral pollen therapy was continued in the Out-Patient Clinic of this hospital under the direction of Captain N. H. Wiley, M. C., and Major M. M. Green, M. C. The same methods have been in use with materials I have furnished.

Major Green has reported the results in 56 patients who have been under observation as follows: Good to complete relief, 36 patients (64 per cent); fair relief, 15 patients

(27 per cent); partial or poor relief, 5 patients (9 per cent). The 1938 pollen season in the vicinity of El Paso was extremely severe, with pollen counts averaging many times those in normal years. Some local physicians believe that this has been the worst hay fever season in the history of this city, with hypodermic therapy much less effective in many cases than in previous seasons. The results with oral therapy during 1938, while not as satisfactory as obtained in previous years, are considered good in view of these circumstances. With an ordinary pollen concentration, the patients having received fair relief might have had a satisfactory season. If this had been the case, the results would have at least equaled those obtained in the two previous years.

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## TRAUMATIC EPITHELIAL CYSTS\*

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TRAUMATIC epithelial cysts seem to occur sufficiently often to make up a pathologic entity which all clinicians as well as pathologists should recognize. Although given various names, such as implantation and epidermoid cysts, the foregoing term seems most suitable, since it indicates etiology, site, and general nature of the lesion. These cysts have been rarely diagnosed as such, but they constitute an important skin lesion that may have interesting complications. It is significant to note that this topic has been given little, if any, space in our textbooks.

Traumatic epithelial cysts were recognized in the middle of the nineteenth century, and numerous articles have been written about them from time to time in foreign literature. The first report in American literature was by Briggs<sup>1</sup> of Boston, in 1895. They were seldom mentioned, however, until the publication of articles by Wien and Caro,<sup>2</sup> of Chicago, in 1934, and Taussig and Allington,<sup>3</sup> of San Francisco, in 1935.

The traumatic origin has apparently been universally accepted, but the method by which injury produces an epithelial cyst is still subject to controversy. Garré<sup>4</sup> was of the opinion that grafts of epithelium were implanted into the subcutaneous tissue by penetrating trauma and formed cysts. Kaufmann<sup>5</sup> experimented with cocksecombs buried in deeper tissues and found that the planted epidermis invariably formed a cyst. Franke<sup>6</sup> was in favor of embryonic cell rests which grew independently and became cystic when precipitated by trauma. Pels-Leusden<sup>7</sup> produced cysts by inserting magnesium disks, which were later absorbed into the corium. He held that the process of epithelialization about such a foreign body arose from cells of the sudoriferous glands and their ducts. The irritant property of a foreign body also produces a type of granulation tissue containing numerous giant cells, a phenomenon which is seen in the wall of some cysts. King<sup>8</sup> gives argument to support the latter theory of genesis. He also stresses the latent period between the injury and the development of the cyst, and favors the term "posttraumatic" epithelial cysts. It would seem that development of epithelium from the sudoriferous glands is the most satisfactory theory.

In the files of the Pathological Laboratory of the Worcester City Hospital we found a total of 37 cases of epithelial cysts from 1930 to 1939, inclusive. Of these, 25 occurred in males and 12 in females; a ratio of approximately 2:1. The

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age range was from 13 to 70 years. The sites were as follows: thumb 4, finger 3, palm 1, forearm 2, shoulder 1, neck 4, cheek 4, orbital region 3, forehead 2, buttocks 2, leg 2, foot 2, and toe 1. No definite sites were given in 6 cases.

Traumatic epithelial cysts are more commonly found in the type of patient exposed to repeated trauma, such as manual laborers, mechanics, and the like. In accord with this, the greater incidence is in males. Some patients develop these cysts from a single injury, which may be severe, or so slight as to be completely forgotten. The common sites are the palms and the flexor surfaces of the fingers and thumbs, as well as the soles of the feet and exposed areas of the face. More rarely they may occur on different parts of the body following skin grafts and certain surgical procedures, such as operative repair of the perineum.

b



Fig. 1.—a, Wall of epithelial cyst, b, keratinized epithelial cells in cyst.

The cysts appear as round or oval swellings, averaging 1 to 2 cm. in diameter, although often reaching greater dimensions. They are firm or semi-fluctuant, usually nontender, and somewhat freely movable. The walls of the cysts vary from 0.1 to 0.3 cm. in thickness. On the inner aspect are attached dense plaques of laminated keratin. The contents are grayish white and greasy or caseous in appearance.

Microscopic section of the walls of these cysts strongly resembles the epidermis (Fig. 1). The outer edge (away from lumen) consists of a single row of conspicuously nucleated high cuboidal cells. This basement membrane may have a few supporting strands of fibrous tissue but no definite capsule. Proceeding inward are several layers of clear polygonal cells. In this stratum the intercellular bridges are usually well marked. Next are a few platelike layers of flattened cells whose nuclei are less distinct and show karyorrhexis with scattered nuclear fragments and small masses of chromatin (Fig. 2). The innermost layer is dead and cornified; the nuclei are absent but the cell outlines are still

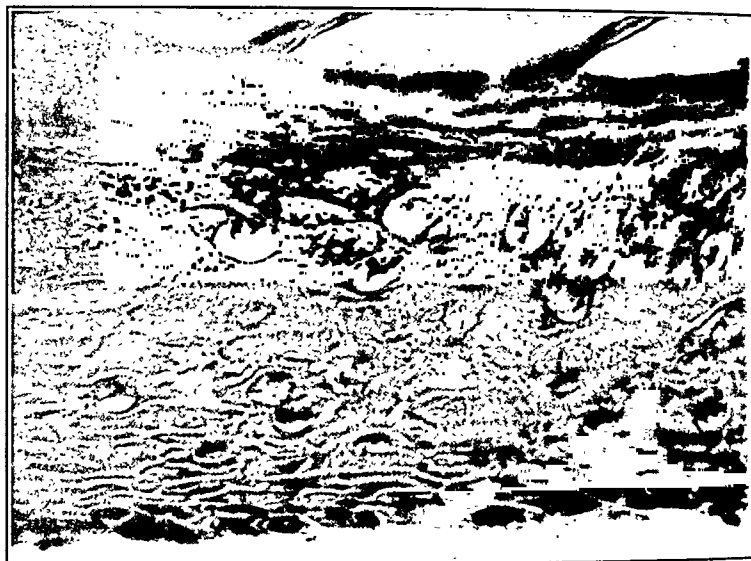


Fig. 2.—*a*, Wall of epithelial cyst showing intercellular bridges and degeneration of cells under the keratinized layer, *b*.

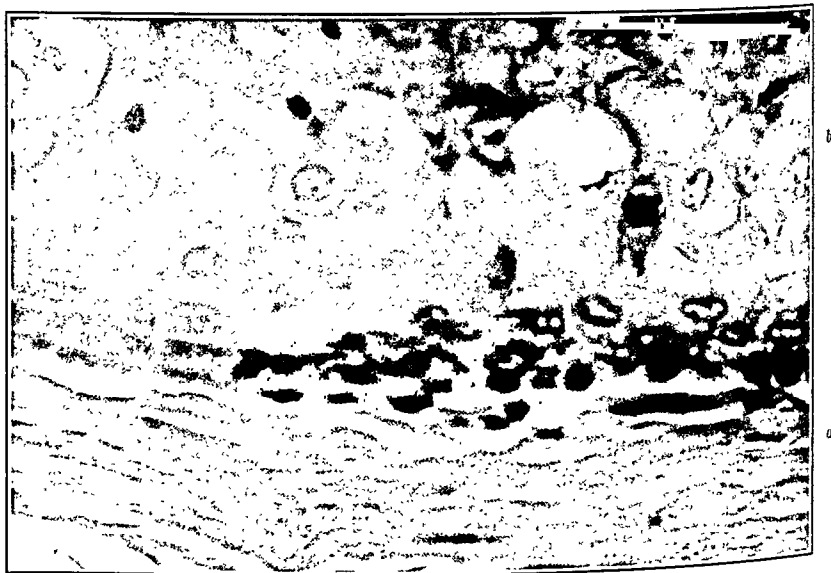


Fig. 3.—*a*, Fibrous capsule on outside of sebaceous cyst; *b*, secretory polyhedral cells without intercellular bridges.

partly visible. The lumen contains keratin and cellular debris. Not all sections of the wall present a typical squamous appearance. Some areas are flattened, as if by pressure, and the thickness varies. The epithelium may be reduced to a thin layer 2 to 3 cells in depth, or it may be completely missing. In such cases there may be small irregular clumps of epithelial cells supported by an increased amount of loose fibrous tissue. Some cysts show foreign body giant cells and cellular reaction with lymphocytes predominating. In comparison, the microscopic characteristics of sebaceous cysts are briefly: a definite fibrous capsule.

absence of distinct layers, absence of intercellular bridges, and an inner layer of active secretory polyhedral cells with no flattening or cornification (Fig. 3).

Traumatic epithelial cysts are most easily confused with sebaceous cysts, but they appear on areas devoid of sebaceous glands or hair follicles. The contents are similar in gross appearance, but the epidermoid cyst does not have the intense "sour" odor often associated with sebaceous material. A rapid differential may be made by smearing the moist contents aided by methylene blue stain. Under the microscope epithelial cells, cellular debris, and keratin are seen, in contrast with the amorphous contents of a sebaceous cyst or wen. Fibromas are solid and often more deeply situated and firmly anchored. Ganglions and synovial lesions communicate with underlying structures and contain thick, glairy fluid. Lipomas and xanthomas are usually easily recognized. Dermoids, articular nodes, and the adenoid epithelioma group of tumors are mentioned only to be ruled out.

These traumatic cysts rarely become malignant and seldom become infected. Since they are usually readily dissectable, treatment is complete excision under local or general anesthesia. If incompletely removed, they tend to recur. An epithelial cyst on the pulp of the thumb or finger may erode into the phalanx.

Two cases that show interesting complications that may develop from traumatic epithelial cysts are cited.

A. G., a 43-year-old male, was admitted to the hospital January 20, 1937, complaining of a painful swollen sole of his left foot of a year's duration. He said he developed a callus over the transverse arch about fifteen years ago, but it gave him no trouble until a year ago, when it grew larger and harder, and he received treatment from a chiropodist. Ten months ago he was given radium treatment at Worcester City Hospital, and following that, cauterization of a sinus tract that broke down and discharged puslike material. Six months ago the patient was sent to another hospital. Examination at that time revealed an area 2 by 4 cm. of extensive keratosis on the left sole over the metatarsus of the second to the fourth toe. The area was tender and surrounded by a small inflammatory zone, but showed no oozing. The patient was given two x-ray treatments and the lesion then excised. It healed with a thick keratotic scar that was somewhat painful. Pathologic report was hyperkeratosis. He was discharged and follow-up treatment was at our Outpatient Department. A few weeks ago there appeared several small elevations about a central depressed area. On pressure there exuded puslike material that proved microscopically to consist largely of desquamated epithelial cells, but no organisms could be found. On admission to the hospital the lesion was radically excised, two toes being sacrificed for skin flaps. The wound healed slowly and the sinuses again became manifest. X-ray showed no evidence of bone infection. Consequently, on the sixty-first day a partial amputation of the foot through the metatarsus was performed. The drain was removed on the third day, and the wound healed by primary union except for a cicatrix in the drainage area. The patient was discharged on the thirty-fourth postoperative day after commencing weight bearing on the foot.

The pathologic report was as follows: Microscopic sections show irregular sinus tracts containing cellular debris and dense masses of keratin. They are lined with stratified squamous epithelium that shows pearl formation but no evidence of malignancy. In the subcutaneous tissue there is polymorphonuclear and plasma cell reaction with areas of necrosis. A few foreign body giant cells are present.

*Diagnosis:* Epidermoid cyst with acute inflammation. The patient made a good recovery, and at the present time he gets around well with a specially made shoe. There has been no recurrence of the lesion.

P. L., a 41-year-old male felt worker, was admitted to the accident floor on January 5, 1935, complaining of a tumorlike swelling of his right thumb. While at work three months before, he picked up a wooden sliver under the nail, which he removed himself. Six weeks later the thumb became swollen and slightly tender. Hot soaks were applied, and later a considerable amount of "pulpy" material was expressed from under the nail. One month later a tumorlike swelling was noticed that continued to increase in size until he was referred to the hospital by his doctor. Examination revealed a firm, rounded enlargement at the end of the thumb, partly under cover of the nail. X-ray showed a clear-cut, crescent-shaped erosion of the tip of the terminal phalanx. Under local anesthesia an incision was made parallel to the nail, and the soft tissue reflected to expose a cavity filled with dry caseous material. A small sac was excised that grossly resembled a sebaceous cyst. The area was cauterized with carbolic acid, followed with alcohol, and a vaseline gauze pack and sterile dressing were applied. Microscopic sections showed squamous epithelium, with excessive keratinization and round-cell reaction.

*Diagnosis:* Epidermoid cyst.

On May 11, 1935, the patient was admitted to the hospital a second time. The lesion failed to heal completely, although the patient was back at work for a time. The tumorlike growth returned and four months later another operation was made on the thumb. The nail was removed and a cystic mass excised. The bone was well curetted and the area carbolyzed. The patient made an uneventful recovery. Pathologic diagnosis was the same as previously. This case also illustrates the industrial compensation aspect of this lesion. The patient has had no recurrence to date.

#### SUMMARY

The importance of traumatic epithelial cysts, also known as epidermoid and implantation cysts, is indicated.

The history and various theories of genesis are briefly reviewed.

Differential diagnosis, especially for sebaceous cysts or wens, is attempted.

Two cases are presented which give interesting complications that may arise from such cysts.

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## STUDIES IN THE ALIMENTARY CANAL OF MAN\*

### X. A ROENTGENOGRAPHIC STUDY OF THE NORMAL PYLORUS AND DUODENAL CAP

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#### PLAN OF STUDY

WHILE there have been many experimental investigations upon the activity of the pylorus and though we are deeply indebted to Lewis Gregory Cole and others for much information upon the human pylorus and duodenal cap, it appeared to us, from our routine observations upon normal stomachs, that there are still many features of interest to be explored in a systematic study of the pylorus by serial roentgenography.

We have not yet acquired a special apparatus, such as that so ingeniously contrived by Jarre,<sup>4</sup> who made studies at such small intervals of time as one second. Since our original studies were made, Gianturco and Alvarez<sup>2</sup> reported serial roentgenographic studies which they made at the rate of four to six per second and Groedel and Franke<sup>3</sup> made similar studies at sixteen per second. Barclay<sup>1</sup> and van de Maele<sup>15</sup> developed and perfected still further this direct method of recording movements in actual roentgenographic series. Reynolds<sup>9</sup> described an indirect method of recording movement which consists of photographing, with an ordinary cinematographic 16 mm. camera, the image of the fluorescent screen. Our method, however, enables us to study pyloric action over longer periods of time than any of the above methods. While it is true that any investigation which is discontinuous is open to objection, we feel that the defects may be theoretical rather than practical, for the mechanism of the pylorus and cap is comparatively slow in action. Our serial roentgenograms were formerly taken at intervals of twenty seconds. This proved unacceptable inasmuch as the rhythm of action may be, though it is not always, of shorter duration than twenty seconds. We, therefore, reduced our intervals to ten seconds and found that, although we do now and again miss a systole of the cap or a pyloric flutter, these omissions can be detected as a rule and discounted.

Naturally the period over which observations can be carried on varies in inverse proportion to the interval between roentgenographic exposures. Our twenty-second interval studies extend over a period of approximately twenty minutes, whereas our ten-second studies last only about ten minutes since we

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limit ourselves to between 70 and 80 exposures in order to keep well within the x-ray skin dosage. The results, as we shall present here, amply justify our effort.

The fundamental features of gastric behavior patterns have already been described in various publications by Todd and Kuenzel,<sup>5-7, 12-14</sup> and summarized in the Beaumont Lectures for 1930.<sup>11</sup> From these writings it is clear that gastric behavior can be very definitely influenced by the type of stimulus employed. There is no difficulty in distinguishing the responses to water, milk, buttermilk, soda, peppermint, heat or cold. In addition the gastric waves have been timed,<sup>10</sup> and it was found that these diverse reagents elicit distinctive responses. The specific effect is transitory, lasting only until the reagent is flushed from the stomach by the flow of gastric juice, a matter usually requiring fifteen minutes. The effect takes time to materialize, and it is usually about three minutes after ingestion before the full specific reaction is attained. Cold is far more evanescent in its effect. A latent period of about a minute and a half occurs as with the fluid reagents and specific activity rapidly rises to a maximum, but the increased vigor of response begins to fade away about five minutes from the moment of ingestion. After the stimulus of heat there is no latent period. Depth, speed, frequency, and vigor of the peristaltic waves are immediately greatly increased. This typical heat modification gradually subsides but, as with our 5-ounce test meals, it may be fifteen minutes before neutral or nonspecific gastric activity is restored.

Since the influences we studied are chemical and not physical in nature, it follows from the foregoing that the period three to fifteen minutes after ingestion is that most suited to our purpose. This is practically covered in the ten-second interval exposure program. We commenced each study two minutes after the fluid was swallowed, so that, when the self-imposed limit of our observation was reached, the specific effect was almost over.

The investigations here recorded cover three phases, namely, opening and closure of the pylorus, systole and diastole of the duodenal cap, and passage of barium from the cap into the descending or second part of the duodenum. When we speak of evacuation, it is the last-mentioned movement to which we refer. The term "passage" in our roentgenoscopic studies means movement of contents through the pylorus, which results in filling of the duodenal cap. This we also see very clearly on the roentgenograms. While semisolid food is doubtless assisted upon its way by peristaltic action, there is ample evidence in the studies previously cited to demonstrate that fluid contents are flushed through the pylorus.

When one of our standard fluid meals is administered, there is immediate continuous passage through pylorus and duodenum, lasting about thirty seconds, after which it suddenly ceases. Apparently the resting stomach has an easily opened pylorus which is caught unawares and closes only after its mechanism becomes aroused. This takes about thirty seconds. Thereafter passage occurs at intervals controlled by the pyloric rhythm and limited by its closure. Enough contents pass to fill more or less the duodenal cap. Systole of the cap itself brings about evacuation into the second or descending portion.

Full evacuation takes place down the duodenum, provided the pylorus is closed when the cap is in systole. If, however, the pylorus is open, systole projects some contents back through the pylorus, and this detracts from the amount of contents which enter the duodenum proper.

With this preliminary statement in mind we can proceed to the discussion of our experimental series in detail. For this study of the normal pylorus and cap we have utilized six experiments (see Table I), our roentgenographic series XII, XV, XVI, XVII, XVIII, XIX, each one of which is arranged as experiment and control so that actually there are twelve studies involved. The six students who served as subjects were thoroughly conversant with the problem and technique. All had been engaged in the conduct of similar experiments. They were sophomores with a year's experience of roentgenographic study. All experiments were made during the morning in late October or early November, 1929, except series XII, which was made during the morning in July of the same year. Detailed notes were recorded at the time of the experiment; this study covers a careful examination of these notes, together with an analysis of the serial roentgenograms made at each date.

TABLE I  
ROENTGENOGRAPHIC SERIES INCLUDED IN STUDY

	SUBJECT	TIME INTER- VAL	CON- TROL	EXPERIMENT	TIME
Series XII (see Fig. 9)	He	20 sec. 20 sec.	Milk	Milk in balloon	10:00 A.M., July 1 10:23 A.M., July 2
Series XV (see Fig. 7)	S	10 sec. 10 sec.	Milk	Soda	10:05 A.M., Oct. 31 10:05 A.M., Nov. 1
Series XVI (see Fig. 8)	P	10 sec. 10 sec.	Milk	Peppermint	10:30 A.M., Oct. 31 10:30 A.M., Nov. 1
Series XVII (see Fig. 5)	M	10 sec. 10 sec.	Milk	Buttermilk	11:20 A.M., Oct. 31 12:20 P.M., Oct. 31
Series XVIII (see Fig. 6)	G	10 sec. 10 sec.	Milk	"Neutral" pattern	11:05 A.M., Nov. 1 12:05 P.M., Nov. 1
Series XIX (see Fig. 10)	Hi	10 sec. 10 sec.	Water	Amyl nitrite	11:43 A.M., Nov. 1 11:51 A.M., Nov. 1

The subject ate his usual breakfast but refrained from all food thereafter, drank no water between breakfast and the control study, and did not smoke, drink, or chew in the interval between this and the time of the actual experiment an hour later. At least two hours, and not more than three, were allowed to elapse between breakfast and the study whether control or experiment. This, as our experience proves, insures an empty stomach when the investigation begins. Swathed only in a sheet the subject was bound lightly to the vertical roentgenographic stand so that his position would remain stationary throughout the study. In all studies except XII, roentgenograms were made at ten-second intervals; in series XII they were made at twenty-second intervals. First the control observations were made comprising 40 exposures or more, according to the plan devised for each study after our



small standard meal was administered. This meal consisted of 4 ounces of vehicle and one ounce of barium sulfate. In the control the vehicle was either milk or water, as dictated by the conditions of the experiment.

In series XVII and XVIII the actual experiment was begun an hour after the control series, for it is our experience that this small standard meal is all eliminated through the pylorus after an hour's time. In series XII, XV, and XVI the actual experiment was begun on the morning following the control experiment at the same time of the morning and under the same conditions. In the actual experiment the subject again took his place as before at the vertical roentgenographic stand and again roentgenograms were made every ten seconds. This time a small standard meal was given, but the vehicle consisted of buttermilk, soda solution, or peppermint, as later described. In the remaining three experiments the second or experimental part of the study was modified, for one study was made of the neutral waves or hunger contractions, one of the effect of amyl nitrite on gastric activity, and one of the effect of a balloon in the stomach. Each experiment had been carried out previously upon the same subject for practice, and the conditions of each experiment were dictated by the experience gained from a study of 10 or more other subjects in an identical experiment.

#### MECHANISM OF EVACUATION

We find in all our gastrointestinal studies that pyloric rhythm seems independent of gastric peristalsis. Evidence for this has previously been presented<sup>11</sup> and it is only necessary, at this point, to recall the conclusion. A particularly strong peristaltic wave may momentarily force the closed pylorus, though this is not a frequent occurrence.

The primary cause of evacuation is cap systole. With an open pylorus, such as is found immediately after swallowing a meal on an empty stomach, the fluid will pour through pylorus and cap in a continuous stream in the absence of cap systole. Again, a vigorous gastric peristaltic wave may force the closed pylorus and momentarily project contents through the duodenum whatever the phase of the cap rhythm. As a rule, cap systole is accompanied by a closed pylorus. This systole results in full evacuation. Should the pylorus not close before cap systole takes place, there is but partial evacuation, for some of the cap contents return to the pyloric vestibule through the open pylorus.

If passage takes place through the pylorus either because it is open or because it is forced by a strong peristaltic wave and the cap happens to be in systole, an extrasystole is superimposed which results in further evacuation. This extrasystole, like the regular systole, appears to be a reflex induced by relative distention.

For the further elucidation of these behavior patterns we have introduced Figs. 1, 2, 3, and 4, which illustrate typical phases of the action. All have been taken from the series comprised in our control study for the effect of amyl nitrite, in which series the stomach contains a standard water meal. In Fig. 1 the pylorus is open, the cap is in diastole and is filling. In Fig. 2 the pylorus



Fig. 1.—Passage through open pylorus into cap in diastolic phase. Water meal. Note the peristaltic contractions in the second part of duodenum. (Series XIX, Hl. No. 23.)



Fig. 2.—Systole of duodenal cap. Water meal. Pylorus closed. Contents passing into distended second part of duodenum. (Series XIX, Hl. No. 26.)

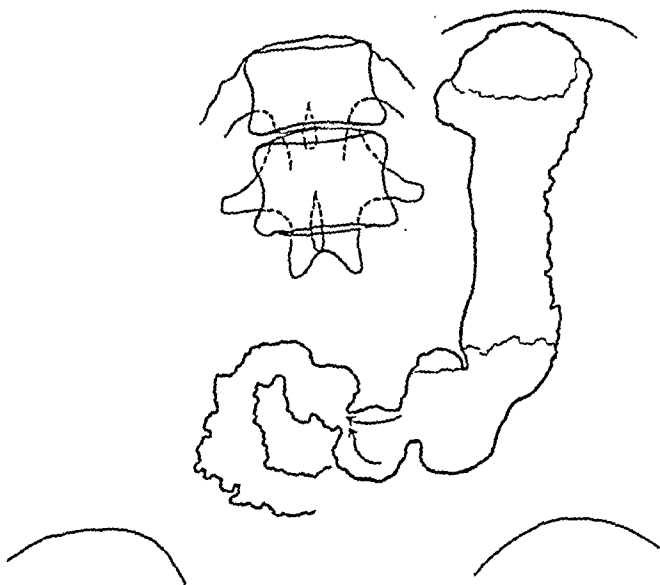


Fig. 3.—Forcing of a closed pylorus by a particularly powerful wave of gastric peristalsis. Water meal. Duodenal cap in systolic phase. (Series XIX, Hi. No. 16.)

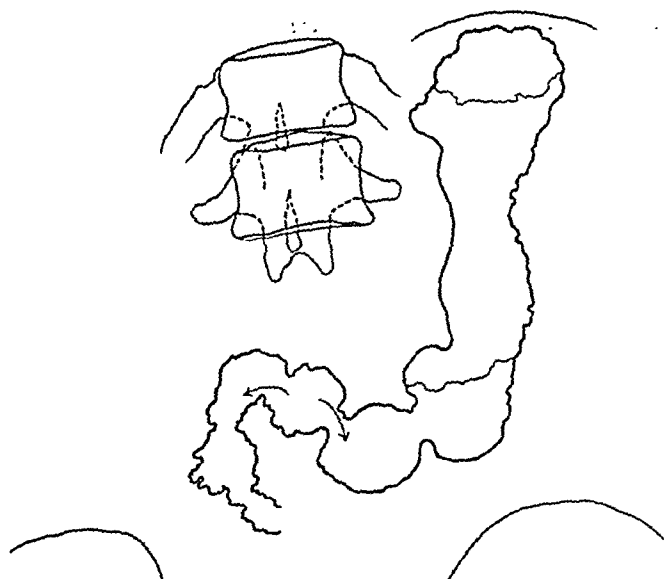


Fig. 4.—Partial evacuation of duodenal cap. Water meal. In a mistiming of cap and pyloric rhythms systole occurs in presence of an open pylorus. Some contents are forced back into the stomach and this reduces the amount of evacuation along the duodenum. (Series XIX, Hi. No. 19.)

is closed and evacuation is taking place into the second part of the duodenum. Fig. 3 shows the forcing of a closed pylorus by an unusually forceful wave of gastric peristalsis, with passage of contents into a cap already in systole. Fig. 4 illustrates the partial evacuation resulting from systole in the presence of an open pylorus. Contents are passing back into the stomach as well as down the duodenum.

## FLUID HEIGHT

The amount of passage and evacuation can be directly checked by observing the fall in the upper level of the column of barium contents in the stomach. We have satisfied ourselves of the essential accuracy of this observation by checking the sinking level in a stomach under normal circumstances and again when the barium contents are enclosed in a balloon (see Fig. 9). In the latter experiment no passage can, of course, occur, and the column level remains constant.

In the graphs an oscillating curve showing fluid height registers the fall in level of barium-containing stomach contents during the period of study. The necessary information is obtained by reading on the roentgenograms the projected distance, parallel with the axis of the vertebral column, between the lowest point on greater curvature of pyloric vestibule and the highest level of the barium-containing fluid in the stomach. The upper delimitation does not necessarily mean the lower border of the Magenblase. In series XVIII, for example (see Fig. 6), the neutral condition of the stomach was investigated an hour after the swallowing of the meal; consequently the fluid level was very low indeed. In series XII (see Fig. 9) the fluid level could not fall since the barium-containing milk was enclosed in a balloon. In series XV and XVI, studies of the effect of gastric regulators (see Figs. 7 and 8), the level remained the same in spite of a continuously patent pylorus and duodenal evacuation. It is difficult to understand how this can happen. Our hypothesis is a profuse gastric secretion which mingles with and replaces the barium vehicle in an active stomach, resulting in a relatively slow passage of the barium salt. In the other studies, where the fluid level falls, we infer that the gastric secretion does not mingle but forms a layer above the barium-containing contents.

## RESPONSE TO MILK

Our series XVII was designed to demonstrate the difference in response to milk and buttermilk. The subject M. was chosen especially because of his equanimity in previous examinations. The first roentgenogram was taken exactly two minutes after swallowing the meal. By that time the regular pyloric rhythm was established (see Fig. 5). Our records do not show absolute uniformity, of course, since they are discontinuous; nor is it necessary to postulate formalism in any way. The pylorus opened for approximately twenty-five seconds and closed for approximately forty seconds during the period of study which lasted seven minutes and forty seconds.

It is only natural to expect that systole of the duodenal cap should coincide with a closed pylorus, and diastole with an open pylorus. In general, this actually happens, but at first there is a little mistiming of the phenomena. The average cap diastole is forty seconds and the average systole is also forty seconds. Examination of Fig. 5 demonstrates the faulty timing of cap systole with pyloric closure. Six minutes after the meal was swallowed, however, the timing became perfect.

Evacuation may be either full or partial and is, as a rule, not nearly so complex in its relationship to the other two phenomena as it is in this particular study. The complication is here caused by mistiming the rhythms in cap and pylorus. We have already discussed the mechanism of evacuation.

Elucidation of the record is, therefore, simple, though it may be tedious. When the first roentgenogram was taken the pyloric complex was confused, probably due to a subconscious disturbance of nervous origin. It is a condition which we have come to expect during the first minutes after the commencement of a roentgenographic study. Three minutes after the meal was swallowed the several rhythms began to adjust themselves. Opening of the

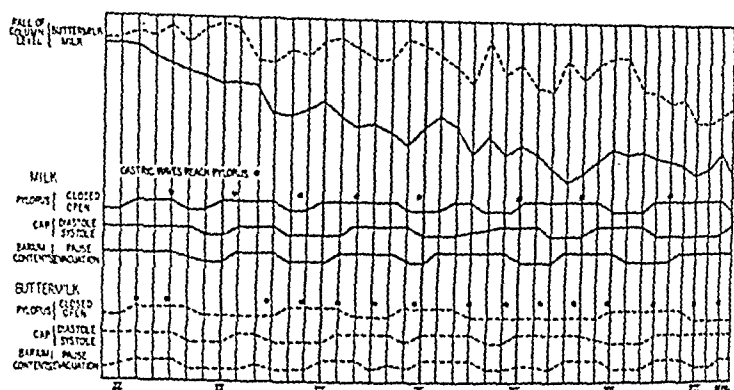


Fig. 5.—Graph of findings in series XVII, M., milk-buttermilk responses. Consult Table I and text for details of experiment.

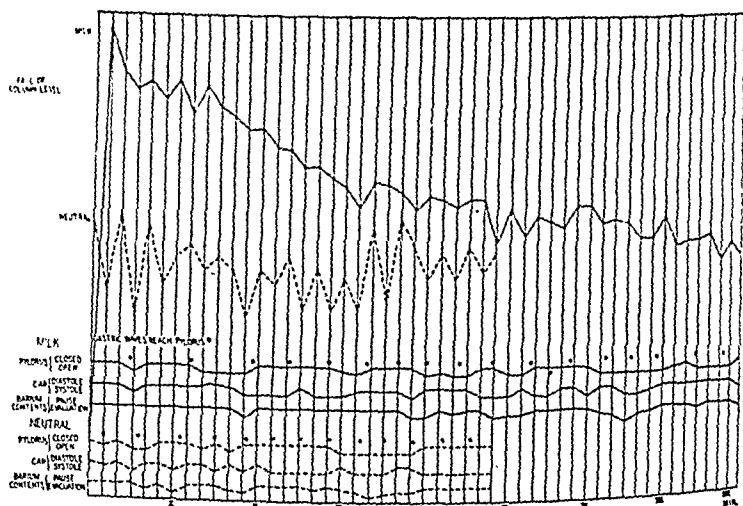


Fig. 6.—Graph of findings in series XVIII, G., milk-neutral responses. Consult Table I and text for details of experiment.

pylorus, with consequent filling of the cap, is followed by cap systole and evacuation. At the end of four minutes the pylorus is again open and systole and evacuation follow. The confusion continues, however, until between seven and eight minutes after the meal is taken. The evacuation shown at this time is partly due to the course of contents through an open pylorus and a diastolic cap and partly due to cap systole in the presence of a closed pylorus. At the end of eight minutes and forty seconds, when the study ceased, the rhythms were in act of establishing their coordination.

Whereas on the average the pylorus is closed for forty seconds and open for twenty-five, cap systole and diastole each occupy approximately forty seconds during this fragmentary study and evacuation follows suit.

Waves of gastric peristalsis still retain their neutral rhythm for a while after the milk is swallowed, but in five minutes the slow milk rhythm is established. Waves follow each other along the stomach at an average interval of forty-four seconds.

Series XVIII, another milk study (Fig. 6), differs from the foregoing in that the first roentgenographic exposure was made just before the meal was swallowed. The pylorus is caught closed which is rather unusual. Within ten seconds from the moment of swallowing, the meal is altogether in the stomach. A momentary opening of the pylorus, accompanied by cap systole, occurs thirty seconds after ingestion. If there was an evacuation at this point, we missed it. A second opening of the pylorus occurs eighty seconds after swallowing. This is followed, twenty seconds later, by systole and evacuation. Very little contents passed the pylorus and, in spite of a systole of half a minute duration, the evacuation lasted but a few seconds. Another systole two minutes and forty seconds after swallowing resulted in no obvious evacuation because the pylorus remained closed. During the entire eight minutes and thirty seconds of observation, the rhythms of pylorus and cap never established themselves in regular synchronization, and evacuation remained erratic. The gastric waves were fairly regular throughout the period and reached the pylorus at intervals of twenty to twenty-five seconds.

The rhythms of the pyloric complex, so far as we observed them, approximated the following on an average. The pyloric rhythm is fairly regular, open for thirty seconds and closed for forty.

The cap rhythm in this study practically synchronizes with the pyloric rhythm at the end of four minutes, previous to which the cap rhythm was a little more rapid. Consequently it does not result in demonstrated evacuation. Cap systole lasts twenty seconds and diastole twenty-five to thirty-five seconds. The occurrence of extrasystoles reduces the average intervals.

Evacuation is apparent after four minutes, when cap and pyloric rhythms become adjusted. Each evacuation lasts about thirty seconds, and there are intervals of forty seconds. Evacuation in this study occurs with cap systole, or when opening of the pylorus causes contents to be pushed through a full cap. If both causes operate together, the evacuation is unusually voluminous.

Examination of pylorus-cap-passage relationships in our third milk study (series XV, Fig. 7) was hampered by the presence of a mucous plug until five minutes after ingestion. This mucous plug is one of the troublesome phenomena of nervousness.<sup>11</sup> It occurred in sophomores during the anxiety complex but was never present in freshmen suffering from the distress or fear complex. In subject S. the plug completely blocked the pyloric canal and distal vestibule so that the fluid column, though fluctuating, never fell until the plug was dislodged when the first evacuation took place and the rhythms of pylorus and cap began to adjust themselves to each other. No gastric peri-

static waves reached the pylorus until this dislodgment occurred. The average interval between gastric waves, apart from this initial period of obstruction, was thirty-one seconds.

After the first five minutes the pylorus opened for an average period of thirty seconds, with intervals of closure averaging twenty seconds.

Cap systole and diastole both extend over thirty seconds. Usually cap systole coincides with a closed pylorus, but if the open phase is long or the cap very full, an extrasystole occurs.

Evacuation lasts thirty seconds and occurs at intervals of thirty seconds.

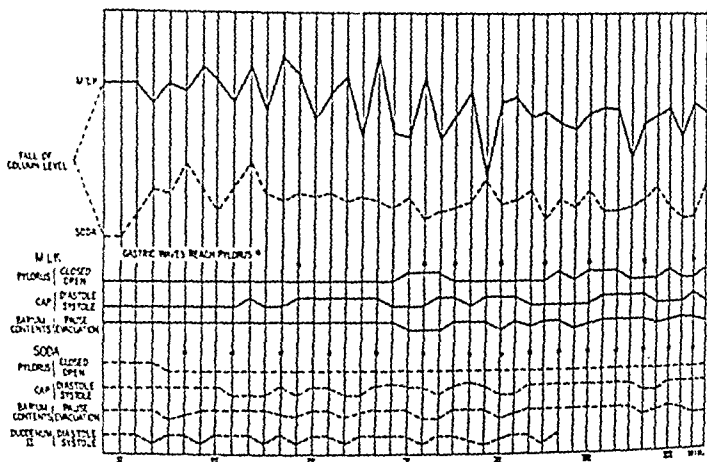


Fig. 7.—Graph of findings in series XV, milk-soda responses. Consult Table I and text for details of experiment.

We made an examination of two more milk studies but were entirely baffled in each series by a mucous plug which did not dislodge during the entire period of observation, lasting almost nine minutes in series XVI (Fig. 8) and thirteen in series XII (Fig. 9). In spite of this the level of the fluid column in the stomach fell as usual so that passage of fluid from the vestibule occurred, though we did not see it.

In summary, then, our five milk studies teach us that milk is not a very satisfactory vehicle for analysis of the pyloric complex. It has no strongly determining influence upon the several phenomena which as a result show marked interference, probably partly of emotional origin. The resultant confusion of rhythms does yield its explanation to intensive study, but the several features are more clearly understood after the far simpler results of the gastric regulators soda and peppermint have been examined.

Eliminating the discrepancies and allowing for interferences of all types, we can safely state that, in the milk pattern, the rhythms of pylorus, cap, and evacuation are strictly harmonious, and that the rhythm is based on units of thirty seconds. This means that pylorus is open for thirty seconds and closed for thirty seconds; that the cap undergoes thirty seconds of systole, followed by thirty seconds of diastole; and that an evacuation, lasting thirty seconds, is succeeded by a pause of thirty seconds once the adjustment of rhythm is established. Gastric peristalsis is also slower after a milk meal than we shall

find it in neutral phase or after soda or peppermint. Consequently milk slows all rhythms and reduces peristaltic vigor, though it does not delay gastric elimination.

#### EFFECT OF GASTRIC REGULATORS: SODA AND PEPPERMINT

Although there are differences in response, the gastric activity induced by the two regulators, soda and peppermint, conforms to a single general plan.<sup>8</sup> Usually not more than two waves are present, visible on both curvatures, extraordinarily slow, deep, steady, regular in rhythm, and so forceful that they appear to nip the shadow almost in two. The stomach shadow indeed often momentarily takes on a trefoil outline. Series XV (see Fig. 7) was designed to record the effect of soda in comparison with that of milk.

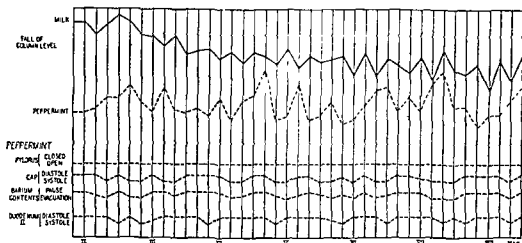


Fig. 8.—Graph of findings in series XV, milk-peppermint responses Consult Table I and text for details of experiment.

The experimental meal consisted of 4 ounces of water, 1 ounce of barium sulfate, to which was added 30 grains of sodium bicarbonate. Series XVI (see Fig. 8) was designed to record the effect of peppermint in comparison with that of milk, and the meal consisted of the usual 5 ounces water meal as above, with the addition of 10 minims of spiritus menthae piperitae. The most unexpected result of administration of both soda and peppermint was the steady maintenance of a patent pylorus. Whereas the pylorus was closed when first observed two minutes after swallowing the soda meal, it opened thirty seconds later and thereafter remained patent. Even at two minutes after the swallowing of the peppermint meal the pylorus was open, and throughout the six minutes and thirty seconds of observation it never once closed.

The elimination of pyloric rhythm was most valuable in our investigation since it left us free to note the relationship of evacuation of cap to the cap rhythm, unhampered by the influence of pyloric opening and closure. There was an evacuation (see Fig. 7) immediately upon the opening of the pylorus after two minutes and thirty seconds. The duodenal cap systole, which occurred at three minutes and ten seconds, produced no registered evacuation, but thereafter, in the presence of a continuously patent pylorus, each cap systole was accompanied by a cap evacuation. Systole lasted on the average twenty seconds, and the period of diastole was thirty seconds. Evacuation, however, occupied only a few seconds. During the first six minutes after



swallowing the meal, the waves of gastric peristalsis reached the pylorus about thirty seconds apart, whereas after six minutes they followed each other at intervals of twenty seconds. In comparison with milk the filling of the cap does not modify its rhythm. Diastole takes thirty seconds in both studies. Soda, therefore, does not modify the cap rhythm. Later it will be shown that peppermint does.

Another marked feature of the response to soda was the clarification of peristalsis in the second part of the duodenum. It was one of our aims to investigate the relationship of gastric and duodenal peristalsis, but we never hoped to find so simple and obvious a demonstration of the duodenal waves. They are recorded in Fig. 7 until between six and seven minutes after the meal was swallowed. Beyond this point the record becomes indeterminate. They are not synchronous with cap systole or with cap evacuation, and they bear no relationship to the gastric waves which apparently stop at the pylorus. In our fluoroscopic examinations we often see duodenal peristalsis, especially after administration of a buttermilk meal. The passage of barium sulfate is characteristically aboral in direction, but sometimes a regurgitant column leaps up toward or into the duodenal cap. Somewhat more frequently there is just an oscillation of barium-containing fluid in the second part of the duodenum. These little streams of "prancing particles" are very striking during the few seconds of their existence.

The average frequency of duodenal peristalsis is one wave every thirty-five seconds; it is, therefore, a little slower than the gastric peristalsis.

After a peppermint meal (see Fig. 8) the pylorus is continuously open after soda, and once again cap evacuation follows cap systole. The frequency of the cap rhythm is hastened by peppermint more than by soda, for whereas, after the latter, diastole still lasts thirty seconds as in our milk studies, peppermint speeds up diastole so that it now continues for only twenty seconds. Both soda and peppermint result in a systole of twenty seconds' duration which is probably a little shorter than that of milk.

In conclusion of our observations on gastric regulators we should point out that the evidence gives us the following information:

Both soda and peppermint in therapeutic doses stimulate gastric activity so that motility is increased and rendered more forceful. There is probably increased secretion which, in consequence of the greater motility, mixes with the barium sulfate and dilutes rather than drives before it the barium vehicle. This action is, therefore, in contrast to that seen after a milk meal.

Both soda and peppermint open the pylorus and keep it open so that the direct relation of cap systole and evacuation may be readily studied. Both have a speeding-up influence upon the cap rhythm, although this is more marked after peppermint than after soda. No distinct shortening of the diastolic phase is demonstrated after soda, although the systolic phase is definitely shortened. After peppermint both systole and diastole are shortened. Later it will be found that these gastric regulators tend to restore neutral rhythms in frequency of gastric peristalsis and in duodenal cap.

Both reagents render duodenal peristalsis clearly visible, and peppermint again is more effective than soda.

Empirical combination of these two for therapeutic purposes is thus given its validation upon scientific grounds.

#### BUTTERMILK PATTERN

After administration of a buttermilk meal the fall in fluid level is slower than after milk, but previous investigations<sup>11</sup> have indicated that buttermilk induces a more profuse gastric secretion. The vigorous motility brings about a thorough mixing of secretion with the gastric contents and so spreads the expulsion of barium sulfate over a relatively greater mass of fluid. Actually it has been found that barium sulfate is *finally eliminated from the stomach* in about equal time both after milk and after buttermilk. The true interpretation of these apparently contradictory phenomena is doubtless to be sought in the dilution of the barium sulfate suspension by gastric secretion, owing to the greater activity of the stomach after buttermilk. On the other hand, following a milk meal, we think the diminished activity fails to bring about a thorough mixture. The gastric secretion lies on top of the barium vehicle and so flushes it through the pylorus with a speed which approximates the loss of barium sulfate to that seen after buttermilk. Five minutes after administration of a milk meal gastric waves become less frequent, but buttermilk waves continue with an unchanged rhythm.

Fig. 5 shows that buttermilk, like milk, fails to establish synchronism in the rhythm of cap and pylorus during the period of observation ending approximately nine minutes after the meal. Its action is far less powerful than that of the regulators. The pylorus is not held open, though its rhythm is slowed down. In the record the average periods of opening and closing each last forty-five to fifty seconds.

The same slowing effect is seen upon the cap rhythm and evacuation, though this effect develops a little more slowly than the change in pyloric rhythm. Cap diastole in this study averages forty seconds and cap systole thirty seconds. With the longer diastolic phase, the cap grows larger than after milk before systole occurs. Evacuations lasting about forty seconds alternate with pauses of about thirty seconds. The comparatively long period of evacuation seems to be due to prolongation of the open phase in the pyloric rhythm. Even if cap diastole occurs during this open phase, evacuation is not stopped, although it is, of course, diminished.

Buttermilk renders duodenal peristalsis visible in our fluoroscopic examinations, but we were not fortunate enough to define its occurrence in this particular study.

In conclusion, buttermilk has a stimulating effect upon gastric activity both secretory and muscular. It slows and regularizes gastric and duodenal rhythms. It has not so powerful an action as the gastric regulators soda and peppermint and its effect is different. Pyloric rhythm, which is influenced before cap rhythm, is slowed but never eliminated. Cap rhythm and evacuation are also affected, diastole and evacuation being plainly lengthened. Duodenal peristalsis, though visible on the fluoroscope, is not so clearly delimited on the roentgenograms as it is after soda or peppermint.

## NEUTRAL BEHAVIOR

An hour after administration of one of our small standard meals there is often still some barium sulfate in the stomach, but the vehicle is completely flushed away. It is not necessary to recapitulate here the evidence upon which this conclusion is based, for the subject has been fully discussed in previous work.<sup>11</sup>

Series XVIII (see Fig. 6) was designed purposely to compare the pyloric and duodenal behavior after a milk meal with that during the neutral phase. The observations at the experimental session, made an hour after administration of control milk meal, lasted five minutes and were sufficient to establish the differences between the two patterns. The presence of a little barium sulfate clinging to the gastric mucosa delineated for us the behavior of pylorus and duodenal cap. Gastric secretion is certainly continuing, though at a minimum rate, and there may possibly be a rhythm in this, since our length, width, and area determinations upon the roentgenograms of this series remain constant while the record of fluid height indicates a five-minute cycle. The waves are a little more frequent than after milk and reach the pylorus at intervals of approximately twenty seconds instead of the twenty-five second interval after milk.

The pyloric rhythm, as in all our studies, is independent of gastric peristalsis, less frequent and irregular. The same characteristics are displayed by the cap rhythm. During the five minute period of observation the two rhythms do not synchronize and they, therefore, produce a confused evacuation. Nevertheless, careful study of our results indicates that the rhythms of both pylorus and cap are essentially in twenty-second phases and that evacuation is a result of systole rather than of an open pylorus. Evacuation is necessarily of short duration since the cap does not fill in diastole. Pyloric opening and closure both last approximately twenty seconds, and this interval is equally characteristic of cap diastole and cap systole.

In summary, the neutral or resting phase of the stomach is characterized by the following features: Gastric secretion is moderate in amount and possibly rhythmic in its volume. Waves of peristalsis (hunger contractions) pass over the stomach and reach the pylorus at intervals of approximately twenty seconds. Opening and closure of the pylorus each occupies about twenty seconds, but this rhythm, which is somewhat irregular, does not synchronize with the rhythm of gastric peristalsis which is very regular in frequency. The cap rhythm also alternates between diastole and systole, each lasting about twenty seconds but, during the period of observation, does not adjust itself to the pyloric rhythm. Since the cap does not fill in diastole, evacuation is of short duration and small.

## WATER PATTERN

Series XIX was undertaken to demonstrate the influence of a water meal and the effect induced by inhalation of amyl nitrite upon this response. In previous work undertaken upon the effect of water, it has been noted that water modifies the neutral pattern very little, and that its effect is of very short duration.<sup>11</sup> The profuse secretion of gastric juice appears to flush water

from the stomach with extraordinary rapidity. We are, therefore, in the habit of commencing our gastrointestinal examinations with a half ounce "meal" of water in which 5 Gm. of barium sulfate are shaken up. This we call an "outliner" inasmuch as some barium sulfate is left clinging to the gastric mucosa after the restoration of the neutral pattern, which occurs about five minutes after the "outliner" is administered. It was essential that we investigate further the pyloric behavior, for its rhythm was so shortened on roentgenoscopic examination that we began to wonder if genuine closure ever really took place. Even after a regular small standard meal of 4 ounces of water with 1 ounce of barium sulfate the neutral pattern is restored in about ten minutes. Since this does not happen till about twenty minutes after administration of any one of our other standard meals to which reference has been made above, the distinction in water effect is very striking.

In series XIX (Hi, Fig. 10) we administered the standard water meal and commenced the serial roentgenography two minutes later. Immediately after the thirtieth roentgenogram had been made, namely, seven minutes after swallowing the meal, a capsule of amyl nitrite was broken in a towel and given to Hi who took three deep inhalations after the thirtieth, the thirty-first, and the thirty-second roentgenograms. Serial roentgenography continued without interruption until the expiration of twelve minutes after the meal was swallowed. At the moment we are concerned with what happened between two and seven minutes after administration of the water.

Pyloric, cap, and evacuation rhythms were irregular until four minutes after administration, though waves of gastric peristalsis established themselves, regularly reaching the pylorus after three minutes. Waves follow each other, as in the neutral pattern, every twenty seconds.

Once the pyloric and cap rhythms are established, namely, four minutes after administration of the meal, they synchronize so that systole accompanies a closed pylorus and diastole an open pylorus. On the average, the pylorus is closed and the cap in systole for twenty seconds, the pylorus open and the cap in diastole for twenty seconds. This is almost the neutral pattern. Evacuation is absolutely synchronous with systole. The activity of the alimentary tract of subject Hi is at all times very vigorous, and it was in this particular study that we were able to examine in detail the passage of barium sulfate through the duodenum and even into the jejunum. In duodenal peristalsis, as in pylorus and cap, the rhythm is very clear. A wave passes over the duodenum every twenty seconds. A further point worthy of note, before we examine the duodenal peristalsis more closely, is the very definite synchronization of a fall in the fluid height with opening of the pylorus. The ensuing rise is probably due to readjustment of stomach walls and secretion of gastric juice. If there were any doubt about this relationship between fall in level with opening of the pylorus, it would be entirely banished by the pause in all activities, including fall of fluid height, following the administration of amyl nitrite.

The progress of peristaltic waves along the duodenum is so clear in this study that we are able to present a complete account of them. They are defi-

nately initiated by cap systole and are visible throughout the duodenum and along the jejunum. If, however, immediately after its regular contraction started by systole of the cap, the second part of the duodenum fills again by regurgitation from below or gush from the pylorus or cap, a small extra wave may start in the second part of the duodenum itself. All waves continue into the jejunum but are naturally more clearly seen in certain areas, owing to the disposition of the bowel and the consequent arrangement of roentgenographic shadows. In this subject the waves occur at intervals of eighteen to twenty seconds and appear on the gut about 60 to 80 mm. apart. The extra wave set up in the second part of the duodenum usually lies between two originating in the cap.

It is not so simple a matter to portray these features in illustrations. In Fig. 1 the cap is in diastole and the peristaltic wave has passed along the second part of the duodenum. On this roentgenogram contents can be seen pouring into the jejunum. In Fig. 2 the cap is in systole and the second part of the duodenum is filling preparatory to the passage of a wave.

The water pattern, as just described, is far more transitory in nature than those of milk, buttermilk, soda, or peppermint. This is due to the extraordinary rapidity with which a profuse flow of gastric juice flushes the water from the stomach.

The several rhythms of pylorus, cap, and evacuation are established four minutes after swallowing a 5 ounce standard water meal, although typical water waves of gastric peristalsis are evident one minute earlier. These are more forceful than neutral waves but of the same frequency. They follow each other to the pylorus at twenty-second intervals. The pyloric, cap, and evacuation rhythms are practically those of the neutral pattern, namely, twenty seconds, though it may be that opening of the pylorus and cap diastole last twenty-five rather than twenty seconds. The three rhythms, however, are far more regular and more closely adjusted to each other than in the neutral pattern, and evacuation invariably follows cap systole.

The fall in height of the gastric fluid level synchronizes with opening of the pylorus and is, therefore, more steplike than in our other studies.

Duodenal peristalsis is very clearly seen after a water meal. It is initiated by cap systole and can be followed into the jejunum. Its rhythm is naturally based upon that of the cap. It is dependent also upon local tension which may be raised during cap diastole by a regurgitation from below or by a gush through an imperfectly synchronized pyloric opening. An extra wave of peristalsis may originate in the second part itself and progress between two regular waves of cap origin. Thus the rhythm of duodenal peristalsis is a little faster than that of the cap.

#### EFFECT OF AMYL NITRITE

So prompt and definite is the action of amyl nitrite that it is regularly used in our roentgenoscopic examination for demonstration purposes. Although, experimentally, Dr. Sollmann informs us he finds it equally effective upon stomach and small intestine, we invariably get a differential effect from thera-

peutic doses used in our student clinic. The effect varies naturally with the dose. If the student is nervous, he takes a sniff instead of an inhalation. The sniff mechanism carries the charged air into the olfactory region of the nose only. Little or no amyl nitrite enters the system, and the experiment will be a failure. We, therefore, find it essential to explain to our students ahead of time the difference between a sniff and an inhalation and caution them that adequate inhalation is necessary for a successful experiment. With the three regular inhalations little or no effect is apparent on the small intestine, though all activity of the stomach ceases within ten seconds of the first inhalation and lasts between two and three minutes.

In this particular study the pylorus happened to be open when the first inhalation occurred. Gastric peristalsis ceased immediately, and no further wave reached the pylorus until two minutes and twenty seconds after the first inhalation. The effect upon the pylorus is not quite so marked. There was a slight attempt at closure between second and third inhalations but this failed, and the next closure of the pylorus appears exactly two minutes after the first inhalation. Duodenal cap rhythm is not so strikingly affected as the pylorus. The systole which was present during the first inhalation gave place as usual to a diastole, and this in turn was followed by another systole after twenty seconds. This systole was but a minor one, and thereafter the cap remained in diastole for one full minute. So feeble, however, were both the cap systoles just mentioned that neither resulted in any observed evacuation. That they probably did result in the projection of some contents into the *second part of the duodenum* is evident by the occurrence of a *lethargic peristaltic wave of unusual character* in the second part of the duodenum ninety seconds after the first inhalation, when the small intestine was evidently recovering from its inhibition. In a previous roentgenoscopic study of this same subject, we observed jejunal and ileal peristalsis active throughout the pause in gastric activity.

Quite a striking feature of Fig. 10 is the interruption of fall in fluid height level, which is apparent throughout the entire two minutes during which gastric and pyloric rhythms were inhibited.

Amyl nitrite then, therapeutically, has an inhibitory effect upon muscular activity, most marked in the stomach but progressively less vigorous on pylorus, duodenal cap, duodenum, and small intestine proper. As one would expect, the inhibition results in an open pylorus, a cap diastole, and inhibition of gastric and duodenal peristalsis. In therapeutic doses we have rarely seen any loss of tone in the stomach and never in the small bowel. Elimination of gastric contents is stayed during the period of gastric inhibition. The effect should last at least two minutes; we have never seen it prolonged beyond three. When activity starts again, the pyloric, cap, and evacuation rhythms do not immediately return in full vigor and speed. These are attained only as the elimination of the amyl nitrite proceeds.

#### INFLUENCE OF A FOREIGN BODY

In the serial roentgenographic study of the response of the stomach to a foreign body, we were not directly concerned with the behavior of pylorus and

cap, and indeed there is but little information in it which throws light on our present problem. Subject He (Fig. 9) had been utilized for the same experiment on many previous occasions. A small standard milk meal was administered to him in the usual manner, but after even more careful preparation than usual. This precaution was not only useless but actually harmful, for it so thoroughly occupied his attention that subconsciously he became extremely nervous, with the result that a mucous plug developed, so voluminous that even at the end of fourteen minutes after swallowing the meal, roentgenograms being taken every twenty seconds, the plug was not yet dislodged.

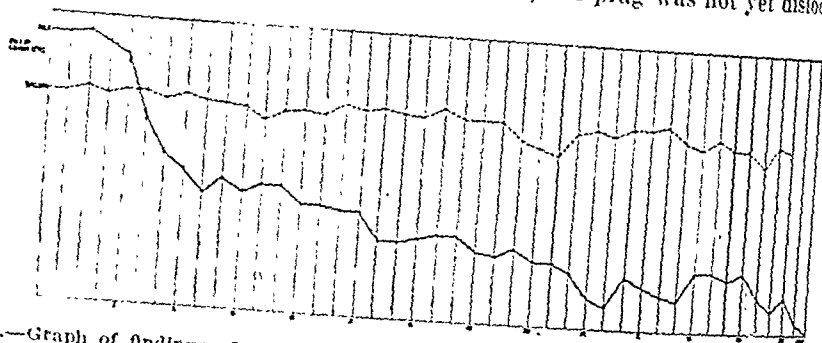


Fig. 9.—Graph of findings of series XII, milk-foreign body (balloon) response. Consult Table I and text for details of experiment.

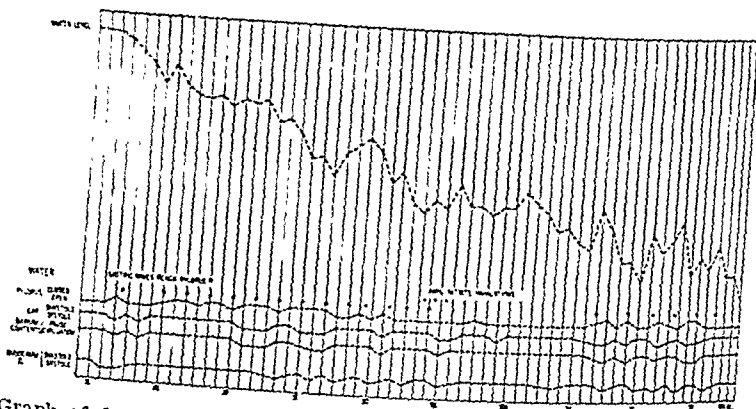


Fig. 10.—Graph of findings in series XIX, water response and effect of amyl nitrite. Consult Table I and text for details of experiment.

Although contents were passed through the pylorus and the fluid column fell in consequence, the faint puffs of milk passages around the plug do not distinctly appear on the roentgenograms. No more can be stated than that the frequency of gastric peristalsis was one wave approximately every thirty seconds.

At the same time on the following day an ordinary condom attached to a soft rubber catheter was swallowed. Ten minutes after it was swallowed a small standard 5 ounce milk meal was poured into it through the catheter. Since this fluid could not escape from the balloon, it is obvious that there could be no passage or sinking of the fluid level. Fig. 9 demonstrates in vivid contrast the fall in the fluid column after the milk meal and the stable upper level characteristic of the balloon experiment.

The balloon, however, stimulates marked gastric motility. A peristaltic wave passes along the stomach every twenty seconds. This is, of course, the neutral rhythm, but the waves are far more forceful than true neutral peristalsis. They catch and nip the balloon in their attempt to force it onto and through the pylorus. The gastric waves do not closely embrace the foreign body because there is, apparently, a profuse flow of gastric juice in an effort to flush away the balloon. These waves are deeper and more forcible than neutral or even water waves. They illustrate the highly unusual stomach behavior pattern induced by a foreign body composed of rubber.

In conclusion, then, a foreign body introduced into the stomach does not alter neutral rhythm but greatly increases the forcefulness of the waves. The contrast between a stationary upper limit of the fluid column in the balloon and the sinking level in the milk control is worthy of close attention.

#### SUMMARY

1. An analysis is made of the roentgenographic records of six specific subjects and their controls, devised to elucidate the normal responses of pylorus and duodenal cap to simple reagents.

2. The reagents used are milk, buttermilk, soda, peppermint, water, amyl nitrite, and a balloon.

3. Pyloric rhythm seems quite independent of gastric peristalsis but is closely coordinated with rhythm of the duodenal cap, although precise adjustment is oftentimes lacking. An open pylorus and cap diastole go together, and a closed pylorus and cap systole. With an open pylorus, passage takes place into the diastolic cap, but a particularly forceful peristaltic wave may force a closed pylorus and expel a little contents into the cap.

4. Evacuation of contents through the duodenum is primarily due to cap systole, but fluid can and does pour through the duodenum in diastole of the cap, provided the pylorus is open. If cap systole occurs while the pylorus is open, some regurgitation takes place into the stomach and a partial evacuation along the duodenum replaces the more typical full evacuation.

5. The second part of the duodenum, like the cap, is sensitive to tension and originates a peristaltic wave itself in the event of regurgitation from below or an extra gush from above. In the latter event, however, the extra-systole is more likely to start in the cap.

6. The neutral or resting phase of the stomach presents a twenty-second rhythm in all motor activities. Gastric and probably duodenal waves recur at intervals of twenty seconds. The pylorus closes, the cap systole occurs, and the evacuation results, taking twenty seconds for their exhibition. Then follow opening of pylorus, diastole of cap, and pause in evacuation, these again occupying twenty seconds. So far as we have evidence there seems to be a five-minute rhythm in the gastric secretion during the neutral phase. The gastric peristalsis, characteristic of this condition of the stomach, is usually defined as hunger contractions.

7. The effect of milk is to reduce the speed of all rhythms from twenty- to thirty-second units or even longer. It also reduces motor activity so that



peristaltic waves are less vigorous or even disappear, to be replaced by a "shimmer" described in earlier work.<sup>11</sup> The reduction of activity and of rhythmic speed does not result in any delay of elimination. Since the milk stimulus is not powerful enough to impose a specific and distinctive pattern on gastroduodenal behavior, there is considerable liability to mistiming of rhythms which complicates the behavior pattern and renders its interpretation difficult, except in the light of our other studies here recorded.

8. Buttermilk, like milk, slows the rhythms of pylorus, cap. and evacuation. It is, however, far more powerful in action. The phases are now of approximately forty-five seconds' duration instead of the neutral twenty-second phase or the milk thirty-second phase. In consequence of the more vigorous control there is somewhat less maladjustment of these three rhythms than after milk stimulation. Furthermore, buttermilk is a powerful stimulant to gastric and intestinal peristalsis. The neutral peristaltic rhythm of twenty seconds is not changed, but the waves are considerably deepened and strengthened so that the profuse gastric secretion is thoroughly mixed with the barium sulfate.

9. Water has a far more transitory effect than the other reagents discussed. Whereas fifteen to twenty minutes must elapse after administration of a standard 5 ounce meal of milk, buttermilk, soda, or peppermint before restoration of neutral rhythm occurs, ten minutes suffices for elimination of water and return to neutral pattern. The profuse gastric secretion apparently flushes out the water. The neutral frequency of gastric and pyloric waves, one every twenty seconds, is maintained, but the waves are much deeper than in the neutral stomach. The regular rhythm of these waves is established within three minutes after swallowing the meal, but pyloric, cap. and evacuation rhythms are not re-established until one minute later. When they return, these rhythms are indistinguishable from the neutral pattern; they retain the twenty-second phases, and there is very definite synchronization. Progress of waves along the duodenum can be very easily studied after a water meal. They are originated by a cap systole, though the second part of the duodenum is also sensitive to tension and may itself originate a wave of peristalsis between two regular waves proceeding from systole of the cap.

After a water meal we can record the synchronization of a fall in gastric fluid height with opening of the pylorus.

10. The gastric regulators, soda and peppermint, are very similar in their action. The waves of gastric peristalsis are extraordinarily powerful after both soda and peppermint. In presence of the latter they may be so vigorous as to give the stomach shadow an evanescent trefoil appearance.

Soda and peppermint both maintain a continuously patent pylorus and neither interferes with the twenty-second rhythm of the waves of gastric peristalsis after the initial period of adjustment which happens in administration of any meal.<sup>11</sup> Cap and duodenal peristaltic rhythms are slowed down by soda, so that diastole lasts about thirty seconds, systole about twenty, and duodenal peristaltic waves follow each other at intervals of about thirty-five seconds.

Peppermint acts in most respects like soda, but there is better synchronization of the various rhythms since the neutral pattern is retained.

11. Our study of the gastric response to the stimulus of a balloon is introduced here solely for the purpose of demonstrating the reliability of our roentgenographic records of the falling fluid height in the stomach. With the milk meal in the balloon there can be no mixture with gastric secretion and no elimination of barium sulfate from the stomach. The fluid height is a reliable indicator of gastric activity. If elimination exceeds secretion, the fluid height falls. If secretion exceeds elimination, the fluid height does not rise, but the shadow width or length increases and the shadow area becomes greater.

12. Amyl nitrite in therapeutic doses has a prompt and definite effect upon gastroduodenal motility for two to three minutes. Gastric peristalsis ceases immediately. The effect is somewhat less marked on pyloric rhythm. Duodenal cap rhythm is also affected, but less than pyloric rhythm. Duodenal peristalsis is rendered lethargic or inhibited for a shorter period than cap rhythm. Jejunal peristalsis is rarely modified. A therapeutic dose is, therefore, far less powerful than experimental evidence suggests, for then intestinal peristalsis is inhibited equally with the gastric waves.

During the inhibitory phase there is no fall in gastric fluid height. Consequently elimination is stayed, but at the same time gastric tone is not reduced.

#### CONCLUSIONS

1. Serial roentgenograms at ten-second intervals provide a ready method of analyzing gastroduodenal behavior patterns.

2. The typical neutral pattern of the stomach is expressed in a peristalsis of both stomach and duodenum wherein the waves follow each other at twenty-second intervals. The pylorus opens and closes every twenty seconds, and evacuation of gastric secretion recurs with every systole.

3. The upper level of the fluid column in the stomach is a good guide to the activity. It falls if elimination exceeds secretion. If secretion exceeds elimination, gastric shadow area is increased through widening or elongation.

4. Water causes profuse gastric secretion; after the initial disturbance all neutral rhythms are quickly re-established, and both gastric and duodenal peristaltic waves are deepened.

5. Peppermint has a similar but far more forceful influence. It, however, maintains a patent pylorus. Pyloric rhythm is eliminated for a time. Other rhythms remain those of the neutral pattern.

6. Soda, like peppermint, greatly increases the vigor of gastroduodenal peristalsis and maintains an open pylorus. Duodenal diastole tends to be lengthened and duodenal peristalsis tends to be slowed. Cap systole and gastric peristalsis retain their neutral rhythms.

7. Milk slows down all rhythms and greatly weakens peristalsis in both stomach and duodenum. It does not, however, hamper gastric elimination, but the retardation of rhythms results in mistiming.

8. Buttermilk still further slows down the rhythms of pylorus, cap, and evacuation, but is a powerful stimulant to gastroduodenal peristalsis. Unlike

milk it does not modify the twenty-second interval between successive waves. It results, like water, in profuse secretion which, however, is less rapidly eliminated and, in consequence of the powerful stimulation of peristalsis, mixes thoroughly with the barium sulfate.

9. Amyl nitrite, in therapeutic doses, has a complete immediate inhibitory effect upon gastric peristalsis and fall of fluid level. It does not reduce gastric tone. It has some, but progressively less inhibitory, effect upon pyloric rhythm, cap rhythm, evacuation rhythm, and duodenal peristalsis.

It is a pleasure to acknowledge the loyal cooperation of the medical students who have lent themselves as subjects for this study, as well as those who have worked with us upon the operating teams. The success of the work has been due to the careful planning and carrying out of the actual studies by Miss Kuenzel, who arranged the experiments and who, with the aid of her assistants, carried them out in precise detail.

The examination and analysis of all the records and the making of the charts are the work of Dr. Painter, the original draft of the manuscript and the deductions are the work of Dr. Todd, and the final preparation of the manuscript for publication is the work of Miss Kuenzel.

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## THE MANAGEMENT OF DIABETES AS CONTROLLED BY TESTS OF ACETONE IN EXPIRED AIR\*

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**I**N DIABETES mellitus there is a disturbance in carbohydrate metabolism characterized by hyperglycemia and glycosuria, and a disturbance of fat metabolism characterized by lipemia and ketosis.

It has been general practice to make tests for acetone bodies in the urine for the purpose of estimating the extent of the ketosis as an aid in diagnosis and control of diabetes, but its management has rested primarily on determinations of glucose in blood and urine. My experience of the past few years indicates that this order of attention might well be reversed; indeed, it appears that diabetes in any stage can be rather well controlled with nothing more than a simple clinical test for acetone in expired air.

Recent studies in the field of "ketolysis vs. antiketogenesis" have tended to alter somewhat our view concerning the significance of the acetone bodies. Heretofore, we have looked on the ketosis as an expression of incomplete combustion of fat which was thought to require for normal combustion a simultaneous vigorous fire of carbohydrate. It now appears that the ketone acids may be normal intermediates of fat metabolism and that the presence of abnormally high concentrations of acetone bodies is simply an expression of intensified fat metabolism. Such an intensification occurs in a compensatory manner whenever the metabolism of carbohydrate is retarded. The ketosis of diabetes may have, therefore, the same significance as the ketosis of starvation and other conditions. However, the subject is not yet settled.<sup>1-3</sup>

From either viewpoint the ketosis would subside as the combustion of carbohydrate is accelerated. There is, therefore, no theoretical disadvantage in basing our control on tests which indicate the extent of the ketosis.

### ESTIMATION OF THE KETOSIS

Analysis of blood from diabetic patients shows all three of the acetone bodies (oxybutyric acid, diacetic acid, and acetone) to be present. And while the fraction of total acetone bodies represented by acetone varies considerably, from 13 per cent to 26 per cent,<sup>4</sup> the determination of this fraction may be taken as a reliable index of the ketosis. A clinical method for determination of blood acetone, more simple than any for blood sugar, has been devised by Abels;<sup>5</sup> this method should find practical application in the management of diabetes.

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Tests for acetone bodies in urine are regarded as more valuable when positive. The prevailing opinion is that they may be absent in the urine even with severe diabetic acidosis;<sup>6</sup> renal block is suggested for the failure of their appearance. That acetone should be present in the blood and absent in the urine seems impossible, since acetone diffuses rapidly through wet tissues without any regard for the biological activities of tissue cells; it is not even necessary for acetone to pass the kidneys to get into the bladder urine.<sup>7</sup> It must be remembered that the more sensitive nitroprusside tests for acetone are actually less sensitive for acetone than for diacetic acid; that possibly with slow rates of urine formation all diacetic acid in the glomerular filtrate might be resorbed by the renal tubules; and that acetone escapes rapidly from warm urine.

Since acetone is excreted by the lungs according to physical laws of diffusion,<sup>7, 8</sup> there is provided in expired air a readily accessible material from which the blood acetone can be calculated and the ketosis approximately estimated. Previous work<sup>7, 8</sup> indicates the distribution coefficient of acetone for blood and alveolar air to be between three and four hundred to one; that is, a liter of alveolar air should contain about as much acetone as 3 c.c. of blood. In the previous report<sup>8</sup> a few observations indicated this relation to hold in diabetes as well as in normal individuals. A few additional observations have been made in connection with this study, which indicate that the blood acetone may be estimated roughly from the relation: acetone in blood = 350 (acetone in expired air). In these experiments alveolar air was obtained by having the patients rebreathe into a rubber balloon; a measured volume was then drawn through 5 c.c. of 5 per cent sodium bisulfite solution. Acetone in an aliquot of the bisulfite solution was determined as in the method of Abels. Acetone in blood plasma, taken at the time of rebreathing, was also determined by the method of Abels. There are two chief errors in this procedure which tend to cancel: the rubber balloon absorbs some of the acetone, and the volume of air as measured is equivalent to a slightly greater volume of alveolar air at body temperature. The ratios of acetone (blood/alveolar air), however, fell consistently within the limits of 300 to 400.

A rapid clinical method for determination of acetone in the breath should be most useful. For this purpose the sensitive Scott-Wilson reagent has been employed by Higgins<sup>9</sup> and others. Turbidities produced from alveolar air obtained by rebreathing into a rubber balloon have been compared with those produced by known quantities of acetone. I have found the ordinary alkaline Nessler's reagent, used by Abels, to be satisfactory. It is nearly as sensitive to acetone as the Scott-Wilson reagent, it is more stable, and it is available in almost any hospital or biochemical laboratory.

Practically, a very good idea of the ketosis may be obtained by an extremely simple procedure. All that is necessary is to have the patient repeatedly blow through a bent glass drinking tube into about 3 c.c. of Nessler's reagent, contained in a large size test tube, until the appearance of a white opalescence. Much of the acetone in the breath passes through the solution without reaction, but the number of blows required to give the test depends

chiefly on the concentration of acetone in the breath and the volume of the expirations. With unconsciousness, as in diabetic coma, the test must be modified in a manner to be described presently.

A normal individual at the end of an ordinary expiration can forcibly expel about a liter of supplemental air, which is chiefly alveolar air. If about 700 c.c. of this supplemental air is expelled repeatedly through the test reagent, pausing about thirty seconds between blows for inspection, then a white opalescence will be observed, usually after the third blow (or the second blow of 1000 c.c.), with blood acetone at the normal level of about 0.25 mg. per cent. This, then, is the state toward which the diabetic patient should strive. A vital capacity spirometer or rubber balloon of known capacity is useful in training the patient to give blows of uniform volume.

#### MANAGEMENT OF CASES WITH THE AID OF THIS TEST

*Severe Diabetic Acidosis.*—If the patient comes in comatose, the diagnosis of diabetic acidosis is confirmed with the aid of Nessler's reagent as follows: The test tube is equipped with a double bored stopper, holding inlet and outlet tubes of glass, and these are connected with short pieces of rubber tubing. The free end of the inlet tubing is placed in the patient's mouth, and suction is applied by the operator on the outlet tubing. A few bubbles drawn through the reagent at the end of three successive expirations gives an intense white cloud in the presence of a severe ketosis. With this test there is no possibility of mistaking hypoglycemic reactions for diabetic coma. This is a point of some importance since tragic mistakes are reported occasionally from the best regulated clinics.

The patient is not catheterized; it is undesirable to superimpose a urinary tract infection, and no necessary additional information is obtained by examination of the urine.

Administration of balanced quantities of insulin and glucose is started at once. Usually 50 units of soluble insulin is administered subcutaneously, and 50 Gm. of glucose, as a 5 or 10 per cent solution in physiologic saline, is administered intravenously.

Progress is indicated by the acetone test which is repeated every half hour. The injection of insulin and intravenous infusion are repeated every hour until clinical improvement is noted, or until the test becomes less intense. With improvement the interval between administrations of insulin and infusion is widened according to the behavior of the individual patient.

With an estimation of the ketosis we have indirectly a measure of the acidosis. Consequently, the determination of plasma bicarbonate becomes superfluous. As a matter of fact, the chief importance of this determination attaches to the calculation of sodium bicarbonate for replacement; this practice has become nearly obsolete with the introduction of sodium lactate, a buffer alkali, which can be administered almost indiscriminately. It is the practice here to administer Hartman's physiologic lactate solution continuously under the skin in various states of acidosis.

As soon as possible the acetone test is obtained by having the patient blow through the reagent. It is carried out frequently and, as long as an

intense cloud is obtained with the first blow, soluble insulin balanced by glucose is administered at the rate of 20 or 30 or more units every six hours.

As the patient recovers from coma and is able to take nourishment, he is ordinarily placed on a calculated diet. The formula being used at present consists of a gram per kilogram each of protein and fat, and 3 Gm. per kg. of carbohydrate; this provides 25 calories per kg. The test for acetone is done night and morning. The use of soluble insulin is continued until the requirements become stationary; the quantity administered being sufficient so that no more than an opalescence appears in the acetone test after the first blow.

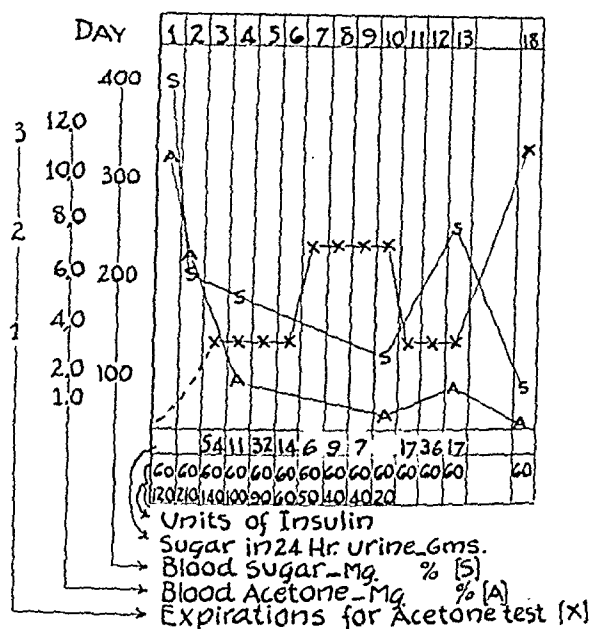


Chart 1.—Observations with severe diabetic acidosis.

A switch to insoluble (protamine) insulin is then undertaken. The practice here is to start out with about 60 per cent of the soluble insulin requirements administered in one injection before breakfast. This change is usually accompanied by a moderate increase in ketosis. A daily increase of 5 or 10 units in the quantity of the injection is allowed until the test for acetone is normal (negative to two, and positive to three blows of about 700 c.c. of supplemental air).

Patients who have been well regulated may develop acidosis with acute infections or break in routine. In such instances protamine insulin, equivalent to maintenance requirements, may be administered each morning, and throughout the day supplementary soluble insulin, balanced by glucose in sufficient quantities to control the ketosis. Observations made on such a case are shown in Chart 1.

This patient, W. J., a negro male, aged 15 years, was admitted January 1, 1933, in a stuporous condition caused by yuletide excesses, and with the onset of an influenzal infection. The test for acetone proved the presence of a severe ketosis. He had been under observa-

tion for some time and was known to require about 60 units of protamine insulin daily; this was administered each morning and supplemented by soluble insulin, as indicated in Chart I. The increase of supplementary soluble insulin from 120 to 210 units on the second day was associated with a rise in temperature and development of delirium. He was able to take fruit juices on being aroused during the first two days and a liquid or soft diet of protein 45 Gm., fat 45 Gm., and carbohydrate 135 Gm. thereafter. On the first day he had 2,000 c.c. of 5 per cent glucose intravenously, and on the second day 3,000 c.c., as supplementary carbohydrate to balance insulin.

Chief interest centered on the expirations required to give the clinical test for acetone in relation to chemical analysis of blood and urine. During the first two days the test was obtained by suction of small quantities of expired air through the Nessler's reagent. At first a few bubbles gave a heavy cloud. This is consistent with the high blood acetone of 11 mg. per cent, and blood sugar of 400. The test from similar small quantities of expired air faded considerably during the next two days, again consistent with blood analysis. The patient was able to blow on the third day, and one expiration gave a rather heavy cloud; the next day, with blood acetone at 2.0 mg. per cent, a light cloud was obtained with one expiration. During the next week, as soluble insulin was gradually withdrawn, the blood acetone remained in the vicinity of 1 or 2 mg. per cent, and an opalescence was obtained from two or one expirations. The patient left the hospital against advice, and five days later returned to the clinic. At this time the test for acetone required three blows of about 600 c.c., and the urine was free of sugar. Analysis of blood (taken four hours after breakfast and 60 units of insulin) gave normal values for sugar and acetone.

Since the amount of expired air required to give the test for acetone bears a definite reciprocal relation to the hyperglycemia, glycosuria, and blood acetone, it would appear that the information gained from chemical analysis of blood and urine is not indispensable.

Where the control is based on levels of blood sugar, a complication arises with the administration of glucose. With the foregoing scheme outlined there is no such concern, and the use of balanced doses of insulin and glucose obviates the danger of hypoglycemia.

Some criticism of this scheme is to be expected from those who feel that glucose is a "poison to the diabetic patient."<sup>10</sup> The use of balanced glucose and insulin is, nevertheless, well supported by clinical and experimental evidence. Elevation of the level of blood sugar without insulin tends to reduce the ketosis,<sup>11</sup> and apparently tends to increase carbohydrate utilization;<sup>12</sup> that is, it tends to correct the diabetic error. In practice, glucose infusions have given good results in diabetic acidosis.<sup>13, 14</sup> I have seen nothing but good results with this scheme; it has been followed on all of our staff patients having diabetic acidosis during the past two years.

*Control of Chronic Cases.*—Previously untreated patients, who continue to exhibit a ketosis with dietary restriction, must be treated with insulin. It seems best to start with a small dose of protamine insulin, 5 to 20 units, depending on the intensity of the cloud produced by blowing through the Nessler's reagent; and then to increase the dose a few units every few days until the test for acetone in expired air is normal. Observations made on a case treated in this manner are shown in Chart 2.

This patient, Mrs. K. W., an elderly white female, came to the clinic because of shortness of breath. She was discovered to have diabetes, and signs of pellagra and heart disease.



She was admitted to the hospital and placed on a diet of protein 45 Gm., fat 45 Gm., and carbohydrate 135 Gm. Various observations made during the following three weeks are shown in Chart 2.

Again one sees the reciprocal relation between expired air required for the acetone test and level of blood acetone. Again the curves for blood acetone and fasting blood sugar are roughly parallel. These points are important because they mean that a patient brought under control by tests for acetone in his breath will be aglycosuric and have a nearly normal fasting blood sugar: features which are generally accepted for the properly controlled diabetic patient. Several other patients have been studied similarly. Always the curves of blood acetone and the fasting blood sugar have been roughly

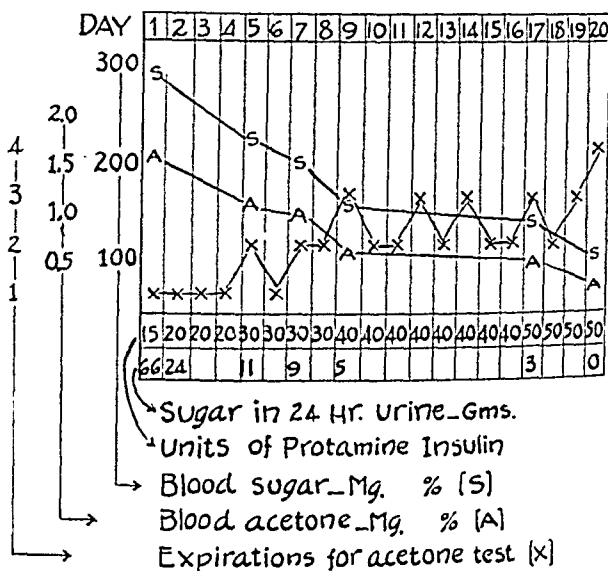


Chart 2.—Observations made during control of a previously untreated patient.

parallel and reduced to normal simultaneously, and always the expirations required to give the test have oscillated about a curve bearing a reciprocal relation to the curve for blood acetone. These oscillations suggest the advisability of refining the test, which could be done by placing the Nessler's solution in an aeration tube connected up with a gas meter or vital capacity spirometer. But the facts are the observed curves for blood acetone and fasting blood sugar have resulted from the use of the clinical test, as described, for control. The daily test, with occasional measurement of supplemental air volume, gives the trend of change in blood acetone.

There has been no difficulty in getting patients to carry out the test at home. They soon learn to empty their lungs after a normal expiration in a fairly uniform manner; this is a factor which must be determined for each individual. They are supplied with a small quantity of Nessler's reagent in a bottle labeled "poison." They are cautioned against drawing the solution into their mouth, and are also told to use vinegar as an antidote if they should happen to do so. The blowing tube is bent so that spray will not be thrown

into the face. A sediment forms in the Nessler's reagent, but the small quantity necessary for the test may be easily poured off from the clear supernatant fluid.

Patients have been told to make the test in the morning before breakfast and to return periodically with a report of the blows required for the test. The time of day at which the test is made, however, is not very important for the reason that blood acetone does not fluctuate during the day as much as blood sugar. For this reason, the patient may be quickly checked in the office. They should be checked, moreover, to determine the volume of their expirations as well as the number required to give the test. The number of blows equivalent to about 2,000 c.c. of supplemental air is taken as the standard for normality.

The information obtained from clinic patients tends to bear out the conclusion drawn from studies in the hospital: the patient who habitually requires two to four long blows to give the acetone test is under very good control.

#### SUMMARY

1. From the standpoint of rationale the control of diabetes may be based on estimations of the ketosis, as well as of the sugar in blood and urine.
2. A simple clinical test is described for the rapid estimation of ketosis.
3. Observations are presented illustrating the changes in blood chemistry associated with control of patients based on the clinical test.

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# THE PHYSIOLOGIC ACTIVITY OF CIGARETTE SMOKE SOLUTIONS AS RELATED TO THEIR NICOTINE CONTENT\*

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IT WAS reported in this JOURNAL<sup>1</sup> that aqueous solutions of cigarette smoke were approximately 25 per cent more toxic by intraperitoneal injection into white mice than a nicotine acetate solution of equal nicotine content. The present study was undertaken with the hope of elucidating this question as well as that concerning the role of nicotine in certain physiologic actions of cigarette smoke solutions.

In all experiments the smoke solutions were prepared by gravity collection over physiologic solution of sodium chloride, using the technique of experimental smoking described by Bradford and co-workers.<sup>2</sup> The solutions were filtered with celite, and their nicotine contents were standardized chemically.<sup>3</sup>

The nicotine solutions used for comparison were made with nicotine acetate in saline. This nicotine salt was selected because in the concentrations used, its solutions in saline have approximately the same hydrogen-ion concentration as the cigarette smoke solution and because it is probably the form in which the nicotine in dissolved smoke largely exists.

*Effect of Cigarette Smoke Solutions and Solutions of Nicotine Acetate on Blood Pressure of the Dog.*—In comparing the effects of smoke solutions and solutions of nicotine acetate on the blood pressure, we followed in many respects the original suggestions of Moore and Row,<sup>4</sup> Ratner,<sup>5</sup> and van Leeuwen,<sup>6</sup> who long ago made similar studies. Carotid blood pressure was recorded with a mercury manometer in adult dogs anesthetized with diall (50 mg. per kg. body weight, intraperitoneally) and to which 0.75 mg. per kg. atropine sulfate had been given intravenously. This atropinization is necessary so as to prevent the initial vagal stimulation which usually follows the intravenous administration of nicotine and which leads to a distortion in the blood pressure curve. Small (0.01 mg. per kg.) initial injections of nicotine were then made to determine the sensitivity of the animal. When the blood pressure appeared normal, the solution to be tested was injected into the femoral vein at a definite rate (0.025 to 0.050 c.c. of a 0.1 per cent dilution of nicotine per second). Injections were repeated at exactly five-minute intervals, at which time the blood pressure had reached a constant level. Further to discount changes in sensitivity (usually a decrease), the following scheme of injecting the solutions was usually adopted in making comparisons: solu-

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tion "A"—solution "B"—"A"—"B"—"A." Statistical evaluation could then be made by averaging the individual responses. To prevent the diminished sensitivity incident to the administration of large doses, such doses of nicotine were selected so as to produce a rise in blood pressure only sufficiently large to permit satisfactory measurement. By observing these precautions, it was found that differences in nicotine content of 25 per cent were, as a rule, readily detectable in any one animal preparation (Fig. 1). Frequently the

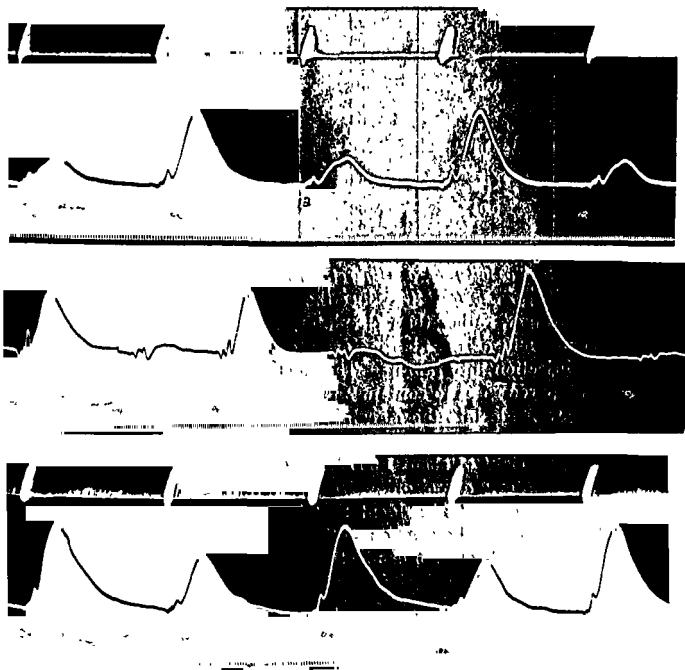


Fig. 1.—Three experiments showing the comparative effect on blood pressure of nicotine acetate in doses differing by 25 per cent, administered intravenously to dogs anesthetized with *hal.* Doses employed in top tracing = 0.026 mg. and 0.0325 mg.  $\times$  kg.; doses in lower two tracings = 0.04 mg. and 0.05 mg. Time six seconds.

difference in the blood pressure rise was proportionately greater than the difference in nicotine dosage. Table I lists the results obtained in several experiments in which the effects of a smoke solution and a solution of nicotine acetate were compared. The similarity in blood pressure response to the smoke solution and to the solution of a nicotine salt of similar nicotine concentration was striking (Fig. 2), and indicates that the effect of the smoke solution was attributable to its nicotine content, as van Leeuwen,<sup>6</sup> and Schlossmann and Schlesinger,<sup>7</sup> among others, had found.

TABLE I

EFFECT OF CIGARETTE SMOKE SOLUTION AND A SOLUTION OF NICOTINE ACETATE ON BLOOD PRESSURE OF DOG

EXPERIMENT NUMBER	DOSE NICOTINE (MG./KG.)	BLOOD PRESSURE RISE (MM. Hg)			
		SMOKE SOLUTION		NICOTINE ACETATE SOLUTION	
		INDIVIDUAL INJECTIONS	AVERAGE	INDIVIDUAL INJECTIONS	AVERAGE
1	0.02	24	20	24	
		16		22	
	0.04	40	45	22	33
		38		60	56
		58		52	
2	0.03	64	58	58	53
		58		58	
		52			
3	0.04	50	50	52	52
		52		52	
		48			
4	0.04	52	50	52	52
		48			

*Intravenous Toxicity of a Cigarette Smoke Solution and a Solution of Nicotine Acetate for the Rabbit.*—Since the mechanism by which nicotine causes death is respiratory rather than circulatory, the toxicities of the solution of cigarette smoke and of the nicotine acetate solution used were compared by intravenous injection into rabbits. The smoke and nicotine acetate solution used were prepared so as to contain 2 mg. nicotine per cubic centimeter. The injections were made into the marginal ear vein of adult rabbits at the rate of 0.2 c.c. per second.\* The animals were of mixed sexes, mostly white, and had an average weight of about 1,600 Gm. Chart 1 illustrates the results produced. The symptoms of poisoning from both types of solutions were identical. From these data it is evident that by intravenous administration into rabbits the toxicity of the smoke solution in terms of nicotine was the same as that of the control solution of nicotine acetate.

*Effects of Irritants and Epinephrine on the Intraperitoneal Toxicity of Nicotine.*—The afore-mentioned results show that by intravenous administration the blood pressure response to, and the toxicity of, the smoke solution appeared to depend entirely on its nicotine content. This being so, the difference in toxicity previously noted<sup>1</sup> between smoke solutions and nicotine acetate solution on mice by intraperitoneal injection, might be regarded as due to difference in rate of absorption. It has been observed here and indicated elsewhere<sup>8, 9</sup> that smoke solutions are much more irritating than similar concentrations of pure nicotine salts. With these observations in mind, the following experiments were performed. In the first test, two solutions of nicotine acetate of the same concentration (0.1 per cent) and hydrogen-ion concentration (pH 5) were prepared. One was made with a buffer solution

\*When fatalities occurred, they resulted usually within a few minutes; however, a few animals in this and subsequent toxicity experiments were kept under observation for several days.

†0.025 normal acetic acid.  
0.025 normal sodium acetate.  
0.025 per cent Benzoic acid.

in a concentration which had been found in this laboratory<sup>9</sup> to have a local irritating effect comparable to that of the cigarette smoke solution. The toxicities of these two solutions were determined on white female mice ( $\pm 20$  Gm. body weight) by intraperitoneal injection. This experiment (Table II, Experiment 1) gave indications that the presence of an irritant could increase the toxicity of nicotine.

TABLE II

EFFECT OF IRRITANTS AND EPINEPHRINE ON TOXICITY OF NICOTINE ACETATE

(White mice—10 mg. per kg. intraperitoneally)

EXPER. NO.	PREPARATION	NO. MICE INJECTED	NO. MICE SURVIVED	NO. MICE DIED	PER CENT FATALITY
1	Nicotine acetate in acid buffer	27	13	14	52
	Nicotine acetate	27	17	10	37
2	Nicotine acetate in 0.056 M formaldehyde	46	17	29	63
	Nicotine acetate	46	26	18	40
3	Nicotine acetate in 1:24,000 epinephrine	66	51	15	23
	Nicotine acetate	66	38	28	42

In a second experiment two additional solutions of nicotine acetate, as described above, were made, except that to one enough formaldehyde was added to make a 0.056 molar solution. This concentration of formaldehyde was selected because it had been found to produce about the same degree of irritation as the buffer solution.<sup>9</sup> These solutions were injected into mice as previously stated. The results (Table II, Experiment 2) demonstrate that the nicotine solution containing the formaldehyde was appreciably more toxic than the one without it. Incidentally, the control injection of ten times this dose of formaldehyde produced no effect, other than a temporary impairment of respiration due to the tremendous distention of the abdomen from the large volume of fluid. From these data we postulated that the local irritation, with consequent vasodilation of the abdominal vessels, lead to an increase in the rate of nicotine absorption, and hence to an increase in toxicity of these irritating solutions. The fact that the solution of nicotine containing a buffered acid irritant was no more effective, might be explained on the basis that its potential acidity was relatively so high that it maintained its acidity longer in the peritoneal cavity than did the plain acetate solution. In unpublished data<sup>10</sup> from this laboratory it has been recorded that nicotine is much less readily absorbed by mucous membranes (vagina) from an acid medium than from a neutral or alkaline one. Therefore, because of the acid medium, the vasodilation due to the irritating properties of this buffered acid was less effective in expediting absorption than a nonelectrolyte irritant, such as formaldehyde.

If the presence of an irritant facilitates the absorption of nicotine acetate from the peritoneal cavity by causing vasodilation, then an agent producing vasoconstriction should have a reverse effect. To test this two more nicotine acetate solutions were prepared, one with plain saline and one with 1:24,000 solution of epinephrine in saline, and their toxicities were compared. The

results shown in Table II (Experiment 3) substantiate the assumption previously made: the solution of nicotine acetate in epinephrine was definitely less toxic than the one without it. Control experiments, in which ten mice were given ten times as much epinephrine as had been given with the nicotine, gave negative results. There is no reason to believe that systemically there is any antagonism between the action of nicotine and epinephrine which might account for a diminution in nicotine toxicity.

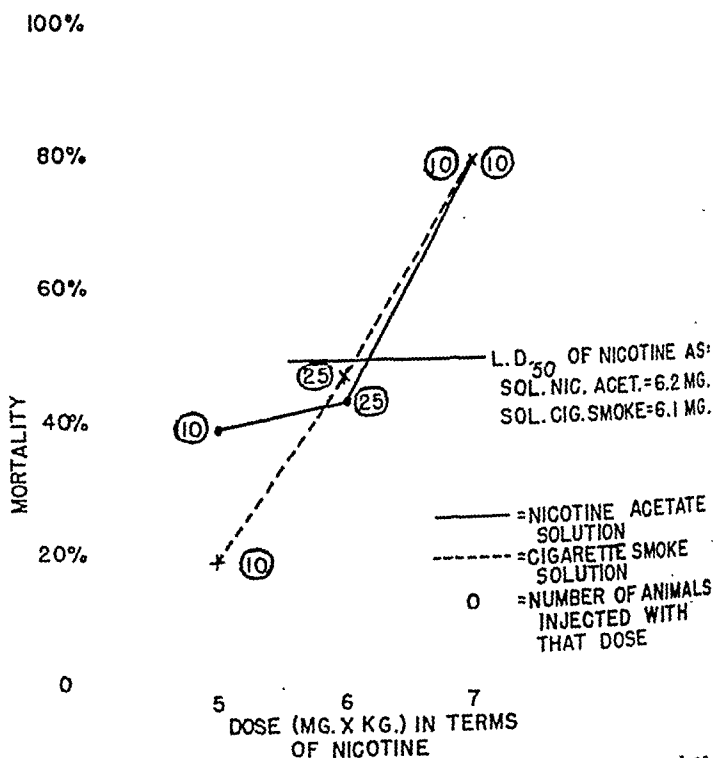


Chart 1.—Showing identical toxicity of nicotine as solution nicotine acetate and as solution of cigarette smoke for rabbits by intravenous injection.

It seems fair to conclude from these experiments that the discrepancy in toxicity between smoke solutions and control solutions of nicotine acetate on mice by intraperitoneal injection is due to quicker absorption of the nicotine from the smoke solution, and that this increased speed of absorption may be explained by a local vasodilation provoked by irritation. These findings at first glance appear to be contradictory to those of Lehmann<sup>11</sup> who found that on human beings the nicotine from smoke was less readily absorbed than volatilized pure nicotine. Cognizance of our findings that nicotine is less rapidly absorbed from an acid medium than from an alkaline one,<sup>10</sup> and recollection that nicotine in smoke is usually combined with acid, answers this seeming point of difference.

*Relationship Between the Physiologic Effects of Cigarette Smoke Solutions and Their Nicotine Content.*—A. *Blood Pressure Studies.* From the foregoing one might conclude that smoke solutions owe their toxicity and effect on blood pressure to the nicotine which they contain. Lee<sup>12</sup> and van Leeuwen<sup>6</sup> distin-

guished between the nicotine content of smoke from several types of tobacco, using as the quantitative criterion the extent of the blood pressure rise provoked in the anesthetized or decapitated cat by the intravenous administration of the smoke solutions. In the blood pressure studies described earlier, it was possible, as stated previously, to discriminate between nicotine solutions differing in nicotine concentration by 25 per cent, and sometimes even less (see Fig. 1). Hence experiments were performed with the intent of determining whether it was possible by such a biological method to differentiate between smoke solutions made from cigarettes of different nicotine contents.

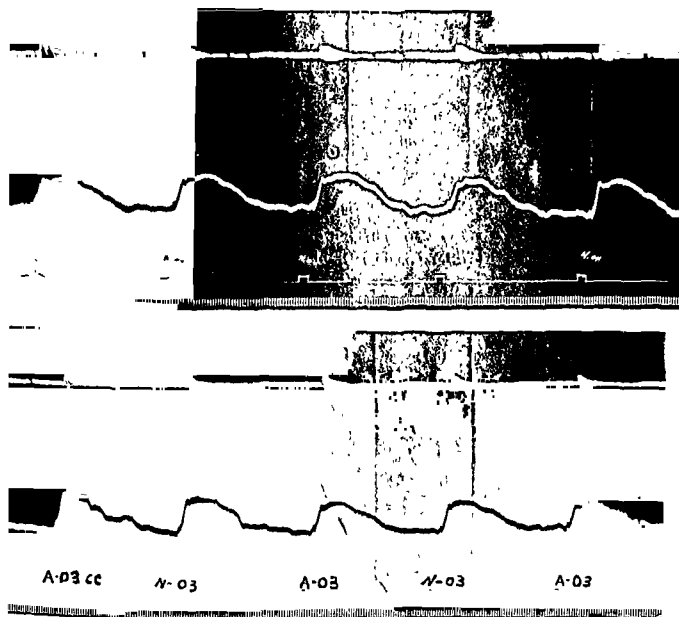


Fig. 2.—Two experiments showing the similarity in blood pressure response to nicotine citrate (0.1 per cent) and a smoke solution of the same nicotine content administered intravenously in equal volumes (0.03-0.04 cc.  $\times$  kg.) to dogs anesthetized with dial. Time six seconds.

Fig. 3 illustrates the results obtained in two typical experiments. Table III lists the actual values obtained in a series of such observations made with smoke solutions prepared from two current brands of cigarettes of different nicotine content.

**B. Toxicity Studies.** Equally, if not more, striking were the results obtained when these solutions were tested for their toxicity on female mice ( $\pm 20$  m. body weight) by intraperitoneal injection. The original smoke solutions were made so as to contain an estimated 0.8 mg. nicotine per cubic centimeter,



TABLE III

COMPARATIVE EFFECT OF SMOKE SOLUTIONS FROM CIGARETTES OF DIFFERENT NICOTINE CONTENT ON BLOOD PRESSURE OF DOG

EXPERIMENT NUMBER	DOSE (C.C./ KG.)	BLOOD PRESSURE RISE (MM. Hg)			
		CIGARETTE A		CIGARETTE E	
		INDIVIDUAL INJECTIONS	AVERAGE	INDIVIDUAL INJECTIONS	AVERAGE
1	0.03	42	44	74	66
		46		64	
				60	
2	0.05	26	26	46	46
		26		60	
	0.07	44	43	56	56
		46		56	
		38		50	
3	0.07	50	43	92	72
		42		68	
		36		62	
				66	

TABLE IV

TOXICITY OF SOLUTIONS OF CIGARETTE SMOKE  
(White female mice—intraperitoneally)

CIGARETTE	A	B	C	D	NICOTINE ACETATE
Nicotine from 40 cigarettes, mg.	108	137	120	110	100
Original dilution, c.c.	135	171	150	137	100
Nicotine, mg. per c.c.	0.803	0.807	0.807	0.803	0.967
Second dilution	83/100	89/100	86/100	90/100	100/100
Mortality, second dilution, 0.015 c.c./Gm. mouse	22/30	16/20	17/20	12/20	9/10
Third dilution	75/100	80/100	80/100	80/100	80/100
Mortality, third dilution, 0.015 c.c./Gm. mouse	6/40	16/40	18/40	19/40	19/40
Dilution for L. D. 50 per cent (interpolated), c.c., 0.015 c.c./Gm. mouse	169	209	185	167	
Toxicity of cigarette	101	125	111	"100"	
Nicotine of cigarette smoke	98.5	125.5	110.0	"100"	11.77
L. D. 50 per cent of nico- tine, mg./kg.	9.6	9.9	9.8	9.9	11.00
Dto. 7/36, male mice (1)	8.5				

based on previous analyses of the smoke. These solutions are designated "original dilution" in Table IV. The "second dilution" was prepared by diluting the original dilution until, when injected in the amount of 0.015 c.c. per Gm. of mouse, there resulted fatalities in more than 50 per cent of the animals. Similarly, a "third dilution" was prepared from the original solution which, when injected in the same amounts, resulted in less than 50 per cent fatalities. The volume in which the smoke of forty cigarettes should be dissolved to give a solution producing 50 per cent mortality when injected in these amounts was then calculated by interpolation from the two points actually determined. The nicotine content of the original solutions was determined chemically, and the median lethal dose (L. D. 50 per cent) of each brand was expressed in

milligrams nicotine per kilogram mouse. The "toxicity" of a brand is the ratio of the L. D. 50 per cent dilution of that brand to the L. D. 50 per cent dilution of one cigarette arbitrarily set at 100. A total of over 700 mice was used for this experiment. Table IV shows the results obtained with the several brands and also with the control solution of nicotine acetate. This experiment demonstrated that the toxicities of smoke solutions prepared from various brands of cigarettes varied directly with their nicotine content. It also demonstrated again that cigarette smoke solutions on intraperitoneal injection into mice are definitely (20 to 25 per cent) more toxic than their nicotine

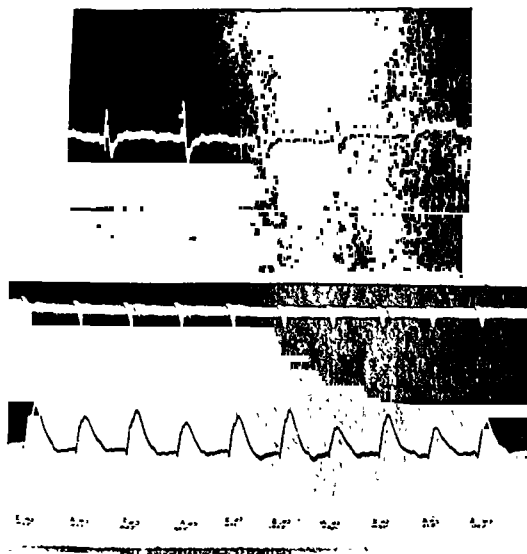


Fig. 3.—Two experiments showing the difference in blood pressure response to the intravenous injection into dogs anesthetized with dial of 3 smoke solutions, each prepared from cigarettes (A, B, E) of different nicotine content. Doses employed = top tracing, 0.01 c.c.  $\times$  kg.; bottom tracing, 0.03 c.c.  $\times$  kg. Time six seconds. One cubic centimeter smoke solution equivalent to 0.37 cigarette of each brand.

content would indicate, as judged from values obtained with pure nicotine acetate solutions. From the experiments on the addition of irritants to nicotine solutions, it would seem that the solution of smoke of greatest concentration should show a lower L. D. 50 per cent for nicotine than the more dilute solution of the same nicotine content. Since, from Table IV, the L. D. 50 per cent for nicotine is the same for all brands, one may surmise that the irritants are approximately proportional to the nicotine content. It is interesting to note that the L. D. 50 per cent value for nicotine acetate is in agreement with that reported by us several years ago<sup>1</sup> for male mice.

## SUMMARY AND CONCLUSIONS

By intravenous administration, cigarette smoke solutions produced the same effect on the blood pressure of the anesthetized dog and had the same acute toxicity for rabbits as a control solution of nicotine acetate of similar nicotine content.

By intraperitoneal administration into white mice, smoke solutions appear to be more toxic than solutions of nicotine acetate of equal nicotine concentration. This difference is accounted for by the more prompt absorption of the nicotine from the smoke solutions due to the presence of irritants which, probably by causing vasodilation, increase the speed of nicotine absorption.

Employing the blood pressure response as a biological test, it was found that the differences in nicotine content in several brands of cigarettes could be correlated with the effects of solutions prepared from their smoke. Likewise the toxicity of these smoke solutions by intraperitoneal injection into mice was proportional to their nicotine content.

It is concluded that the actions of cigarette smoke solutions, as regards their acute toxicities and their effects on blood pressure by intravenous injection, are due to their nicotine content.

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# THE EFFECT OF SUBCUTANEOUS INJECTIONS OF CRYSTALLINE INSULIN ON THE BLOOD SUGAR OF FASTING RABBITS\*

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SAHYUN and Blatherwick<sup>1</sup> were first to demonstrate the difference in the duration of action of insulin on the blood sugar of rabbits following the various modes of its parenteral administration. Thus it was shown that insulin subcutaneously injected has a more prolonged effect than insulin injected intravenously or intraperitoneally. Hence it was considered desirable to investigate whether or not the rate of absorption and the duration of insulin action on the blood sugar of rabbits would be influenced by different modes of subcutaneous injections. Three different subcutaneous routes were studied: (1) subepidermal, (2) subdermal, and (3) intradermal.

While considerable work has been done on the prolongation of insulin action, little attention has been given to the acceleration of its hypoglycemic effect. This is important, particularly in cases of diabetic acidosis and coma, where an immediate lowering of blood sugar is highly desirable. In this connection data is presented to show that the acidity of a solution of insulin has considerable influence on the rate of its absorption.

This study deals with an investigation on: (1) the effect of different modes of subcutaneous injections of insulin on the blood sugar of rabbits, and (2) the acceleration of insulin action by changing the acidity of its solution.

The solution of crystalline insulin used in this investigation was prepared from dry insulin crystals which had been assayed at 22 international units per 1 mg. Sahyun's method<sup>2</sup> for blood sugar determination was employed.

## EXPERIMENTAL

(1) *Effect of Different Modes of Subcutaneous Injections of Insulin on the Blood Sugar of Rabbits.*—In this series of experiments, a solution of crystalline insulin containing 40 units per 1 c.c. was used. Three different modes of subcutaneous injections of insulin were studied on rabbits that had fasted for twenty-four hours. (1) *Subepidermal.* This procedure consisted in shaving a small area of the rabbit's ear, inserting the needle diagonally, and injecting the insulin so as to form a small lump under the epidermis. (2) *Subdermal.* This is the most commonly used procedure of subcutaneous administration of insulin. It consists in giving the desired amount by inserting the needle below the dermis and into the fascia. (3) *Intradermal.* This consists in selecting a suitable area on the back of the rabbit, removing the fur by cutting it close

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to the skin, inserting the needle diagonally into the dermis to a depth of about 1 mm., and slowly injecting the insulin.

The effect of insulin given by each of the subcutaneous modes was studied on 24 rabbits. Each rabbit received a total of 2 units of a 40 units per 1 c.c. concentration. Samples of blood for sugar determination were withdrawn at 0, 1.5, 3, 5, and 7 hours, respectively. The results of these experiments are summarized in Fig. 1.

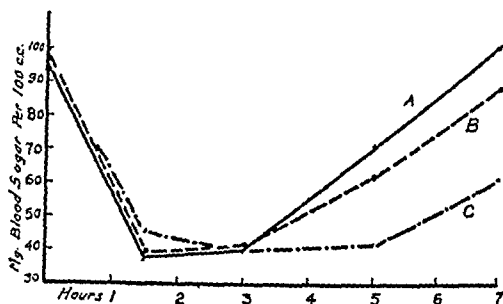


Fig. 1.—The effect of subepidermal, subdermal, and intradermal injections of a solution of crystalline insulin on the blood sugar of fasting rabbits. A, subepidermal; B, subdermal; C, intradermal.

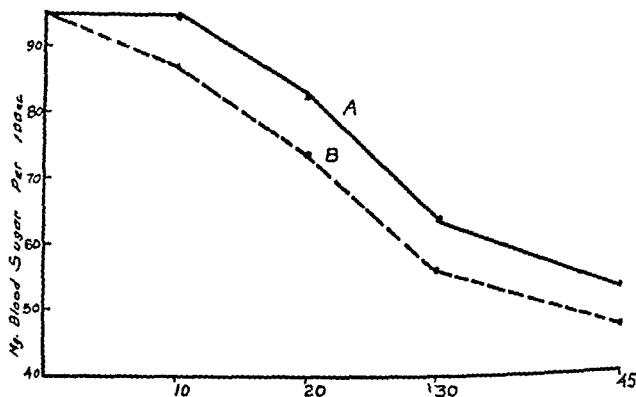


Fig. 2.—The effect of subdermal administration of solutions of crystalline insulin at pH 2.9 and at pH 7.0 on the blood sugar of fasting rabbits. The blood sugar curves from the first hour to the end of the sixth hour were almost identical and were, therefore, omitted. A, crystalline insulin pH 2.9; B, crystalline insulin pH 7.0.

(2) *Acceleration of Insulin Action by Changing the Acidity of the Solution.*—A solution of crystalline insulin containing 40 units per 1 c.c. was prepared from dry insulin crystals. The acidity of the solution was adjusted with dilute hydrochloric acid to pH 2.9. From this solution two dilutions were prepared: (A) 20 units per 1 c.c. dilution was made in the usual manner and the acidity kept at pH 2.9, and (B) 20 units per 1 c.c. dilution was prepared by the addition of an isotonic phosphate buffer so that the final acidity was pH 7.0. The isotonic phosphate buffer was prepared as follows: 18.9 Gm. of disodium hydrogen phosphate were dissolved in about 200 c.c. of distilled water, the acidity adjusted to pH 7.3, and the volume brought to exactly 1 liter. A careful check of the pH of the final 20 units per 1 c.c. dilution was made after mixing equal amounts of the buffer and the solution containing 40 units per 1 c.c.

After the withdrawal of the initial samples of blood for sugar determination, one-half the number of rabbits were subdermally injected with 1 unit per kg. body weight of preparation A (pH 2.9) and the other half with 1 unit per kg. body weight of preparation B (pH 7.0). Subsequent samples of blood were withdrawn at ten, twenty, thirty, and forty-five minutes, and one, two, three, four, five, and six hours, respectively. The following week the experiment was repeated and the animals that had received preparation A received preparation B and vice versa. Each preparation was tested on 24 rabbits that had fasted for twenty-four hours. The results of this experiment are recorded in Fig. 2.

#### DISCUSSION

The data presented in Fig. 1 clearly demonstrate the wide variation in the duration of insulin action following its administration by the different subcutaneous routes described, viz., intradermal, subdermal, and subepidermal.

For all intents and purposes the duration of hypoglycemia caused by the injection of insulin may be divided into three periods:

(1) *Period of Activity*: This period extends from the time insulin is injected until the time the sugar content of the blood has reached its lowest level. The duration of this period is from one and one-half to two hours. During this period the blood sugar is decreasing at a faster rate than it is being mobilized from the liver.

(2) *Period of Coma*: This period marks the height of the activity of insulin and its duration varies with the kind and amount of insulin injected as well as with the mode of administration and the experimental animal. It covers the time when the blood sugar has reached its lowest level and continues to be so until the rate of glucose mobilization from the liver is in excess of the removal of sugar from the blood. During this period the experimental animal is in a coma and convulsions may occur.

(3) *Period of Recovery*: This period is characterized by the rise of sugar in the blood until it reaches its initial level. It also marks the vanishing effect of injected insulin. In most instances the slope of the curve assumes a straight line.

Following the subepidermal injection of insulin into the rabbit's ear, there appears to be very little storage of insulin at the site of injection. Apparently all the insulin (2 units) administered has been rapidly absorbed, so that as soon as the blood sugar has reached its lowest level, which marks the end of the first period, it begins to rise. The second period is of a very short duration. Owing to the rapid absorption of insulin by this method of injection, the blood sugar falls to a lower level than when it is subdermally or intradermally injected. Within seven hours the blood sugar returns to its normal level.

Following the subdermal administration of insulin, the duration of effect of insulin is somewhat parallel to the subepidermal route. However, the period of recovery is slower, as characterized by the slope of the curve in Fig. 1 and the fact that at the seventh hour period the blood sugar has not reached its initial level. One may, therefore, conclude that the subdermal administra-

tion of insulin causes it to be more slowly absorbed than when it is given subepidermally in the ear.

The intradermal administration of insulin into the same group of rabbits has indeed yielded unexpected results. The action of insulin thus given has been considerably prolonged, as characterized by the length of the second period. Furthermore, at the end of the seventh hour the blood sugar is still very low. The prolongation of action of insulin by this mode of administration may be due to the formation of a depot caused by the lack of proper circulation in the vicinity of the site of injection. This difference in action suggests that when insulin is rapidly absorbed, as in the case of subepidermal or epidermal injection, a portion of the activity is lost, either by inactivation of the insulin or by stimulation of the adrenals, with a greater mobilization of sugar from the liver.

Another striking difference is illustrated in the effect of the same dose of insulin administered subepidermally, subdermally, and intradermally on the incidence of convulsions. Table I is a self-explanatory summary.

TABLE I  
INCIDENCE OF CONVULSIONS

ADMINISTRATION ROUTE	NUMBER OF RABBITS		AVERAGE TIME FOR OCCURRENCE OF CONVULSIONS (MINUTES)
	USED	CONVULSING	
Subepidermal	24	7	131
Subdermal	24	7	103
Intradermal	24	15	265

Following the subepidermal and subdermal administration of insulin, the animals that went into convulsions recovered without the administration of glucose. Convulsions occurring following the intradermal injections were severe and in some instances the rabbits died despite the administration of ample quantities of glucose. Death, as a rule, occurred at night after the apparent recovery from hypoglycemic coma.

Since most investigations of insulin action, particularly on the retardation of its effect, have been done following subdermal injections, it was considered desirable to employ this procedure in these studies on the acceleration of its effect.

The first period, termed the *period of activity*, may further be divided into two stages: (1) inactivity or equilibrium stage and (2) activity or absorption stage. In an earlier publication from this laboratory<sup>3</sup> it was pointed out that when an acidified solution of insulin was subcutaneously administered, the insulin must pass through various stages of hydrogen-ion concentration until the reaction at the site of injection is in equilibrium with that of the tissue fluid. During this period of equilibrium there is no lowering of blood sugar. The data presented in Fig. 2 support this theory, for it is clearly demonstrated that the subdermal injection of an acidified solution of insulin remains inactive for the first ten minutes or thereabout, whereas, when the acidity of the solution of insulin has been adjusted to about that of the tissue fluid, the lowering of blood

sugar occurs promptly. The acceleration of effect of such a preparation persists throughout the first period of activity. A rapid-acting insulin of this nature may be useful in such instances as diabetic coma and acidosis.

#### SUMMARY

Intradermal injections of a solution of crystalline insulin were shown to produce a more prolonged hypoglycemia and a greater incidence of convulsions in rabbits than subepidermal or subdermal injections. The lowering of blood sugar in fasting rabbits following the subdermal administration of insulin is more prompt when the acidity of its solution is at pH 7.0 than when it is at pH 2.9.

The author wishes to express his appreciation to M. Goodell and A. Nixon for their technical assistance.

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# LABORATORY METHODS

## PHOTELOMETRIC DETERMINATION OF INORGANIC SULFATE IN BIOLOGICAL FLUIDS

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**N**EPHELOMETRIC determinations of sulfate in biological materials have been reported by various investigators, especially by Denis,<sup>1</sup> and Denis and Reed.<sup>2</sup> In view of the recent development of the photoelectric cell as a biological instrument, it seems worth while to report a procedure adapted to its use.

The Cenco-Sheard-Sanford photelometer<sup>3, 4</sup> was employed with a micro absorption cell No. 12337. A deep yellow filter was used to increase the sensitivity. To obtain the standard curve from which future values were to be read, the following procedure was employed: To a series of seven 12 c.c. test tubes, containing from 0 to 6 c.c. ammonium sulfate solution equivalent to 11.45 gamma sulfur per c.c. (diluted from a nitrogen standard containing 1 mg. nitrogen per c.c.), water was added to make up to 6.6 c.c. To this solution 0.4 c.c. 20 per cent sulfate-free trichloroacetic acid was added and stirred with a fine glass rod. One cubic centimeter gelatin at about 40° C. (just above melting) was added and the contents stirred. After ten minutes 2 c.c. 1 per cent barium chloride were added and vigorously mixed with the solution. After a ten- to thirty-minute interval the barium sulfate cloud formed was read in the photelometer. The time intervals stated were found optimal for giving reproducible results. The tube containing no standard was used as a control to detect the presence of any contaminating sulfate. If a cloud was obtained, the set of determinations was discarded.

Table I presents the amounts of reagents necessary for each tube and gives the average readings and the standard deviation of the mean for each dilution of sulfate over a series of 20 experiments. The curve given in Fig. 1 was obtained from plotting the average readings for a series of 20 experiments against the sulfur, expressed in gamma, contained in each tube of solution. This curve, plotted on semilogarithmic paper, approaches a straight line except in the region of greatest concentration, where, according to the standard deviation of the mean, it becomes unreliable.

*Modification of Procedure for Determination of Inorganic Sulfate in Serum.*  
*Preparation of Protein-Free Filtrate.*—To 2 c.c. serum in 15 c.c. graduated centrifuge tubes, 1.4 c.c. 20 per cent sulfate-free trichloroacetic acid are added.

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This solution is thoroughly mixed and allowed to stand fifteen minutes for the protein to flocculate. After centrifugation the clear supernatant liquid is filtered through a dry acid-washed filter paper into a test tube. The wall of the centrifuge tube is washed with 2 to 3 c.c. of water, added to the supernatant liquid in the filter. Dilution is then made with water so that 21.1 c.c. in all are added, bringing the total volume of the diluted serum to 24.5 c.c.

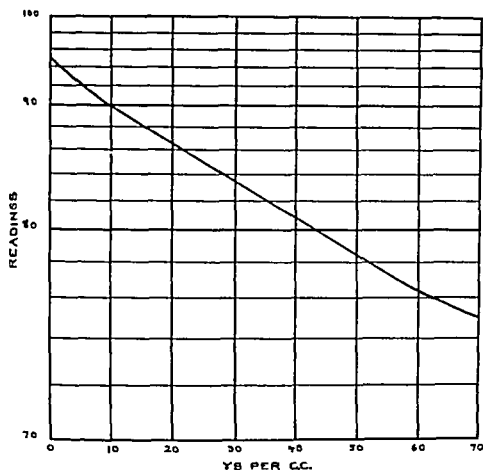


Fig. 1.—Photometric determination of sulfate. Total averages of readings for series of 20 experiments.

TABLE I  
PHOTOMETRIC DETERMINATION OF INORGANIC SULFATE

STAND. C.C.	H <sub>2</sub> O C.C.	20% CCl <sub>3</sub> COOH C.C.	GEL. C.C.	1% BaCl <sub>2</sub> C.C.	AVG. READINGS FOR 20 EXPTS. γ S	STANDARD DEVIATION OF MEAN γ S
0	6.6	0.4	1	2	94.79	0.52
1	5.6	0.4	1	2	89.41	0.68
2	4.6	0.4	1	2	85.77	0.47
3	3.6	0.4	1	2	82.46	0.75
4	2.6	0.4	1	2	79.59	0.84
5	1.6	0.4	1	2	76.89	0.75
6	0.6	0.4	1	2	75.34	3.90

*Determination of Sulfate in Filtrate.*—Transfer 7 c.c. of this diluted filtrate to the small-sized test tube used in the sulfate determination, and add 1 c.c. gelatin and 2 c.c. 1 per cent barium chloride ten minutes apart as previously described. After a ten- to thirty-minute period the barium sulfate cloud formed is read in the photometer.

There are two controls for each set of determinations. One, which contains the filtrate and gelatin but no barium chloride, is used to determine a

cloud which may already be present in the solution due to a slightly cloudy filtrate. If this is present, its value must be subtracted from the reading obtained for the barium sulfate cloud. A cloudy filtrate is avoided if care is taken not to disturb the protein precipitate when rinsing the walls of the centrifuge tube. In no case in the series of experiments recorded was a cloudy filtrate encountered.

The other control used is identical to the one used in the sulfate determination to detect the presence of any contaminating sulfate. This contains 6.6 c.c. water and 0.4 c.c. trichloroacetic acid instead of 7 c.c. of filtrate.

Calculation for the inorganic sulfate is made in terms of mg. sulfur per 100 c.c. of serum as follows:

$$\frac{R \times 24.5 \times 100}{7 \times 2 \times 1000} = R \times 0.175$$

R represents the reading on the curve. 24.5 equals the total volume of diluted serum; 7 equals cubic centimeters filtrate; 2 equals cubic centimeters serum taken; 100 equals cubic centimeters serum in which the final value is expressed. 1000 translates gamma to milligrams sulfur.

TABLE II

PHOTOMETRIC DETERMINATION OF INORGANIC SULFATE IN SERUM, INCLUDING ALSO TWO DETERMINATIONS IN PLASMA  
(VALUES EXPRESSED AS S PER 100 C.C. SAMPLE)

SAMPLE	SULFATE MG.	SULFATE ADDED MG.	SULFATE RECOVERED MG.	PERCENTAGE ERROR	
1	0.902	0.286	1.303	+ 9.6	Serum
2	1.303	0.286	1.536	- 3.3	Serum
3	1.269	0.573	1.703	- 7.5	Serum
4	1.303	0.573	2.104	+12.2	Serum
5	0.902	0.573	1.403	- 4.9	Serum
6	0.868	0.573	1.303	- 9.6	Serum
7	0.668	0.573	1.303	+ 5.0	Serum
8	0.935	0.573	1.403	- 7.0	Serum
9	{0.902	0.573	1.336	- 9.4	Plasma
	{0.902				Serum
10	{0.735	0.573	1.202	- 8.0	Plasma
	{0.701				Serum

Table II gives a series of determinations of inorganic sulfate in serum, showing also the amount of sulfur added in the form of ammonium sulfate in each case and the sulfate recovered. The determination of inorganic sulfate in two samples of plasma, obtained from citrated blood, is also recorded with a comparative determination in serum from the same specimens. As shown in the table, the error varied from + 12 to - 10 per cent.

This method may also be employed to determine the inorganic sulfate content of other biological fluids, such as tissue extracts. If the sulfate content is too great to be read on the plotted curve, a dilution of the protein-free filtrate may be made with a solution of trichloroacetic acid (1 part trichloroacetic acid to 16.5 c.c. water). The chief value of this method lies in the ease and speed with which determinations may be made. It has proved especially useful in working with tissue extracts under experimental conditions in which changes of sulfate content are of the order of several hundred per cent and a

number of unknowns are carried out simultaneously. Its accuracy is only slightly less than more laborious methods.

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## SIMPLIFYING SERIAL DIAGRAMS FOR MEDICAL FORMS

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GRAPHIC descriptions of the progress of various anatomic lesions have long simplified the compiling of results in various medical series. As a rule, however, the expense of having a series of special cuts made and the printing charges discourage such treatment of some experimental studies and the results are likely to lose some of their clarity. For this reason, a method was sought to simplify the cut problem and yet produce clean cut, identical serial diagrams.

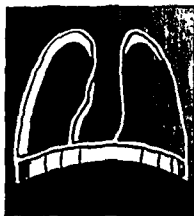


Fig. 1.

After a sketch has been made of the organ or region that is to be duplicated—prostate, larynx, heart, lungs, etc.—and the size determined, a metal cut is made by bending strips of  $\frac{1}{8}$  inch tin into the proper shape and soldering them upside down to a flat piece of tin mounted on a wood block. It is then placed against the flat side of an emery wheel to level the cutting edges, after which the edges are filed quite narrow.

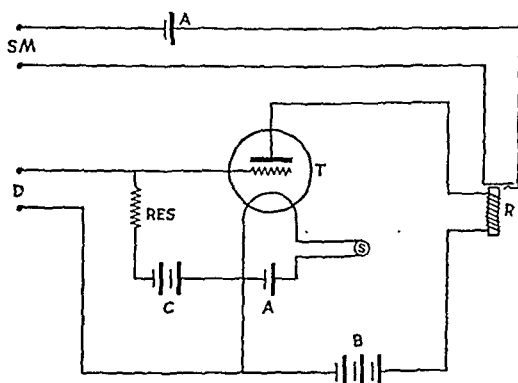
The form is now outlined and typed on a mimeograph stencil, and impressions are made in the proper places by tapping the cut several times with a hammer.

These impressions are much cleaner and more exact than line drawings on a stencil. The cuts also have the added advantage of use for odd reports when inked with a stamp pad and a blotter placed under the paper.

## A SENSITIVE DROP RECORDER\*

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THERE are several different methods for recording the flow of liquids when the rate is sufficiently slow to permit the liquid to fall from the outlet in drops. Gibbs<sup>1</sup> has presented the essential features of the different types of drop recorders. The "Universal Mains Drop Recorder," described by Winton,<sup>2</sup> makes use of a triode valve. It is sensitive but has the disadvantage of having one of the terminals of the signal magnet connected to the 110 volt current supply. There are possibly modifications in use where this is not the case, but we failed to find descriptions in the literature.



WIRING DIAGRAM for DROP RECORDER,  
 A -  $1\frac{1}{2}$  Volt Dry Battery.      R - 1000 Ohm Advance Relay.  
 B - 45 Volt Radio B Battery.      S - Switch.  
 C -  $4\frac{1}{2}$  Volt Radio C Battery.      T - Type 31 Radio Tube.  
 D - Terminals for Drop Contacts. SM - Terminals for Signal Magnet.  
 RES - 3 meg. Resistor.

Fig. 1.

With the help of two medical students, Mr. Sam Leslie and Mr. Noble Wynn, who are amateur radio enthusiasts, a recorder was built according to the diagram shown in Fig. 1. The parts can readily be obtained from any good radio supply house and are comparatively inexpensive. The entire apparatus can be mounted in a small metal cabinet and thus facilitate moving. Due to the use of batteries there is no danger of shock, such as is the case with the higher voltages. A very small amount of current flow between the "Drop Contacts" is necessary to actuate the relay and consequently the recorder can be used with very dilute solutions of electrolytes. In the case of viscid secretions or fluids that may change in viscosity during the course of an experi-

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ment, the method of displacement of a salt solution, described by Gibbs,<sup>1</sup> should be employed.

The drop contacts described by Owen,<sup>3</sup> Biskind and Dan,<sup>4</sup> and Gibbs<sup>5</sup> can be used with this apparatus. We use the arrangement given in Fig. 2. The necessary metal parts are mounted on a piece of bakelite, 6 by 15 by 65 mm. The actual contacts are of 22 gauge platinum wire and should be pointed or bent slightly upward in order to assure a positive contact with each drop.

Any standard signal magnet designed to operate with 1½ volts D. C. can be used, or the battery in this circuit can be changed to permit the use of any signal magnet.

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### SPECTROCOLORIMETRY: ADAPTATION OF A COLORIMETER FOR EVALUATING ABSORPTION SPECTRA\*

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INVESTIGATION of physiologic and pathologic pigments by means of the usual type of comparison spectrometer is frequently unsatisfactory because of difficulty in controlling light intensities and in varying the concentrations of test solutions. Furthermore, the dispersion of the spectrum does not provide sharp absorption bands with those concentrations of abnormal pigments encountered clinically. A further disadvantage for the small laboratory is the cost of the instrument.

To secure a comparison spectrometer which would be practical under routine conditions, a few alterations were made in a Duboseq colorimeter so that there would be no interference with its regular use, yet would permit its rapid and simple change into an instrument for observing and quantitatively measuring absorption spectra. In practice its usefulness has so far exceeded expectations that its description for the benefit of others seems warranted.

Although the principle is by no means new, specific details are not readily available. The spectroscope of the Krüss spectrophotometer,<sup>1</sup> which presumably can be applied to any colorimeter, provides a wave-length scale and permits slitting down to a restricted region of the spectrum—both of which are important for certain types of precise analysis. Watson<sup>2</sup> mentioned employing a "double spectroscopic attachment for a colorimeter" upon which

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the manufacturer, at the present time, can furnish no information. Weiss<sup>3</sup> used similar equipment except that it possessed a wave-length scale. The apparatus to be described is not intended for highly precise spectrocologic measurements, but is entirely suitable for use in the clinical laboratory for the purposes specified.

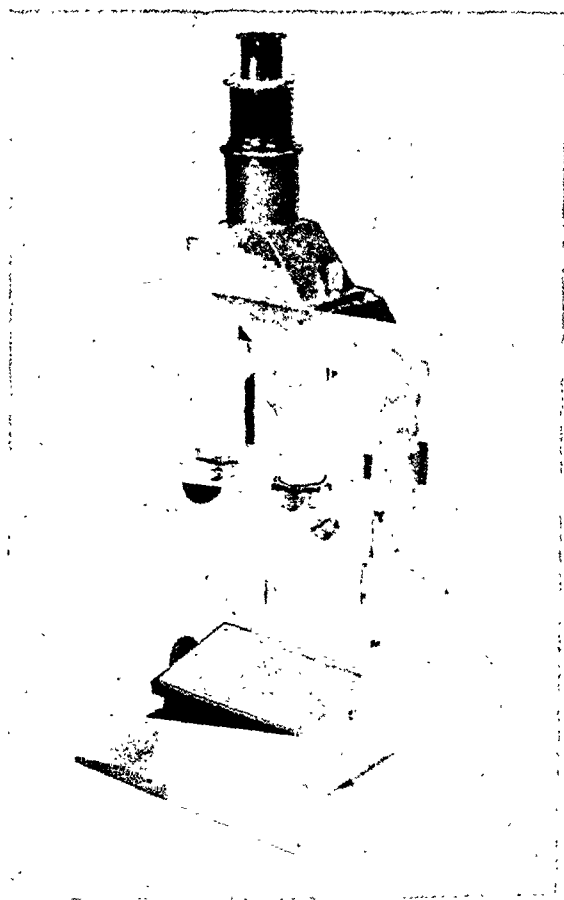


Fig. 1.

#### MODIFICATION OF THE INSTRUMENT

The feasibility of the desired alterations depends upon either the utilization of material at hand or the selection of new equipment which can most easily be adapted to several uses. The spectrocologic requires a fairly large field, such as that provided by the Bausch and Lomb colorimeter. Breadth of spectrum likewise is a necessity not offered by many hand spectroscopes. Experimentation with different models indicated that the most satisfactory was the direct vision spectroscope listed by Bausch and Lomb as No. 33-80-75. Its short length (72 mm. with the draw tube not extended) permits its insertion in place of the colorimeter ocular, where it is held by a supporting collar permanently attached to the spectroscope. By means of the draw tube, the collimating lens can be focused upon the slit (0.1 mm.) which is ruled on a silvered glass disk and is not adjustable. For the type of analyses described

later, it is felt that a fixed slit is imperative. The apertures are protected by cover glasses from dust and corrosion. The spectroscope is rotated into a position where the spectrum is bisected throughout its length from red to blue, thus allowing the absorption bands of a specimen placed in the right hand cup to be compared with a similar solution on the left. The instrument is convertible from a spectrophotometer to a colorimeter by lifting out the spectroscope and replacing the regular eyepiece. Such adaptability has proved to be a great convenience in routine laboratory work.

The old-type, five-piece colorimeter cups required more fluid for comparison than was usually available. Moreover, greater depth of fluid was desirable than could be secured with the cups alone. The *hollow*, dark glass plungers and cups of the Klett colorimeter were ideal for our purpose and had the advantage of being stock equipment in the laboratory. Acquisition of new Bausch and Lomb fittings for the old instrument would have entailed considerable expense and would not have provided the right type of plungers. Since the plungers on the Bausch and Lomb colorimeter were much larger than those from the Klett, a reducing bushing was made to hold them. To accommodate the Klett cups, the holes on the colorimeter stage had to be enlarged slightly.

#### USES FOR THE SPECTROCOLORIMETER

*Measurement of Porphyrin Concentrations.*—In the original Dobriner method<sup>4</sup> a Goudsmit-Summerson photoelectric colorimeter<sup>5</sup> was used with a filter allowing the passage of light at 5,320 to 6,000 angstrom units. Subsequently, Dobriner<sup>6</sup> discarded the colorimeter and employed a hand spectroscope to study the intensities of the absorption bands in a series of known and unknown solutions. This comparison was made in small test tubes by sheltering them against the light from the side while making spectroscopic observations down through the solution. Obviously, inspection of both standard and unknown at the same time, rather than shifting from one to the other, would be a decided improvement from the standpoint of convenience as well as precision. Use of a comparison spectrometer made this possible but required tedious adjustment of the tubes to obtain equal illumination and sharpness of absorption bands. The spectrophotometer, which has been described, rendered the comparison as simple as the use of an ordinary colorimeter.

The stock standard, 1 mg. of coproporphyrin in 100 ml. of 5 per cent hydrochloric acid, or a suitable dilution thereof, is adjusted in the instrument to match the unknown extract. In the event of abnormally high concentrations, the latter may require dilution of the final 5 ml. volume to 10 or 15 ml. with 5 per cent hydrochloric acid. Either solution may be set at 20 mm. and the other varied until the intensities of the absorption bands are equal on both half fields. Although comparison is slightly more difficult than with simple color matching, remarkably close agreement in readings is obtainable.

Three bands are possible, but only two are to be anticipated: a dense line in the green and a much weaker one in the yellow. The third may occasionally be seen as an exceedingly fine line about midway between the other two. With a concentration of 10  $\gamma$  per 5 ml., the two bands are readily discernible. At 5  $\gamma$  per 5 ml. only the more dense band is visible through 40 mm. of fluid.



Below this concentration the band cannot be seen unless the hollow plungers are filled with solution also, thereby increasing the depth by approximately 50 mm. When so used, it is imperative that the plunger wall be more or less impervious to light or that an efficient shielding device be employed.

The stock standard keeps well in the refrigerator if preserved with a few drops of chloroform. When diluted with acid for comparison with normal urine specimens, its keeping quality is diminished. Attempts to use *Hematin-Nordmark* for the preparation of standard solutions were unsatisfactory, not only because of difficulty in securing known concentrations, but also because of dissimilarity of the absorption bands. Urine extracts, once evaluated against the stock standard, have been refrigerated and used thereafter as known solutions.

*Detection of Abnormal Blood Pigments.*—Although it is possible to obtain a rough estimation of hemoglobin concentrations by matching diluted specimens against known solutions, the method is not practical. The spectrophotometer is exceedingly useful, however, in distinguishing between methemoglobin and sulfhemoglobin. With the advent of sulfanilamide and related types of chemotherapy, the existence of sulfhemoglobin has been widely heralded. Only recently has it been generally recognized that the bands characteristic of methemoglobin and sulfhemoglobin lie so close together that confusion in their identification may readily occur. Although the difference between these two pigments has been adequately described in the literature,<sup>7, 8</sup> separate viewing of the absorption spectra is not always convincing. With the spectrophotometer, however, it is apparent to the most casual observer that the characteristic band of sulfhemoglobin is definitely nearer to the yellow region of the spectrum, while that of methemoglobin extends further into the red.

It is impractical to specify a fixed dilution for examining blood for abnormal pigments since the concentration of hemoglobin in pathologic specimens varies so widely. The dilution should be such that it is possible to blot out the passage of light through the spectroscope when the solution is at its greatest depth in the colorimeter cup. It is rare indeed that methemoglobin reaches so high a concentration that its band in the red can be observed at the same time that the alpha and beta bands of oxyhemoglobin are separately seen. Sulfhemoglobin, on the other hand, is not infrequently detectable under these conditions.

When the blood is abnormally dark in color but examination with a spectroscope fails to reveal a band in the red, the spectrophotometer can be used to advantage to demonstrate the presence of methemoglobin or sulfhemoglobin. A sample of normal blood, suitably diluted, is placed in the left-hand cup and adjusted to a level which shows narrowing of the red portion of the spectrum by encroachment of shadow from both sides, but more particularly from the deep red. With abnormal pigment, gradual increase in the depth of fluid in the cup leads to development of a definite shadow at the yellow end of the red field. This shadow extends further into the red for methemoglobin than for sulfhemoglobin. Gradual blotting out of all light reveals marked differences between oxyhemoglobin and these abnormal derivatives.

The difference between the absorption spectra of carboxyhemoglobin and oxyhemoglobin is so slight that their distinction in the spectrophotometer is not to be anticipated except where an extraordinarily high concentration of the monoxide derivative is encountered. In the presence of 80 per cent carboxyhemoglobin differentiation is readily made, but the characteristic color of this pigment is equally apparent to the unaided eye. Detection of carboxyhemoglobin in the blood by spectroscopic means cannot be expected clinically in those cases where the concentration is consistent with life.

*Application to Congo Red Tests.*—In determining blood volume by means of injected Congo red, it is possible for considerable hemolysis to exist without detection. This source of error is even more pronounced when this dye is used for the diagnosis of amyloid disease. It is a simple matter, however, with the spectrophotometer to introduce the solutions into the colorimeter cups and examine them for the characteristic bands of oxyhemoglobin. Congo red itself in the concentration used absorbs no light in this region. Once hemolysis is ruled out, the spectroscope is replaced by the colorimeter eyepiece and the test completed as usual.

#### SUMMARY

1. Mechanical changes necessary for the conversion of a Duboseq colorimeter into an instrument for observing and measuring absorption spectra have been described.

2. The adaptation is economical since permanent attachment of the spectroscope is not required and the colorimeter is not altered so as to affect its routine use.

3. The instrument is particularly well suited to the quantitative estimation of urine and fecal porphyrins.

4. The difference between methemoglobin and sulfhemoglobin is readily demonstrable with the spectrophotometer. This is true of carboxyhemoglobin and oxyhemoglobin only in excessively high concentration.

5. Both detection of hemolysis and determination of Congo red in blood volume and amyloidosis tests are possible with this instrument.

After completion of this paper it was discovered that Boyd and Myers (*J. Lab. & Clin. Med.* 13: 1043, 1928) had employed a "spectrocomparator" embracing the principles described herein for the purpose of determining urobilin.

We are greatly indebted to Dr. Konrad Dobriner for providing us with the stock solution of porphyrin used in this study.

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## THE ESTIMATION OF SERUM PHOSPHATASE ACTIVITY WITH DISODIUM PHENYL PHOSPHATE\*

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KING and Armstrong<sup>1</sup> introduced the use of disodium phenyl phosphate for the estimation of the activity of serum phosphatase. This substrate is advantageous because it is more rapidly hydrolyzed by the enzyme than the more commonly employed sodium beta-glycerophosphate. Furthermore, the liberated phenol, which has a greater chromogenic value, is more susceptible to ordinary colorimetric estimation than is the orthophosphate liberated from sodium beta-glycerophosphate.

This paper presents a revised technique of the method of King and Armstrong<sup>1</sup> for the determination of serum phosphatase. The technique was devised to increase the accuracy, simplicity, and clinical adaptability of the procedure.

The important changes that have been introduced are as follows:

1. The serum protein is removed from the sample by precipitation with trichloroacetic acid after incubation and before the liberated phenol is estimated colorimetrically. The presence of the serum protein is undesirable because of the presence of the tyrosine residues in the protein molecules, which produce a considerable amount of color with the phenol reagent. Further, serum protein produces high values for the control tubes and makes the estimation of the phosphatase units in the sample less accurate. Also, it may cause turbidity when the phenol color is developed with sodium carbonate according to the directions given by King and Armstrong.

2. To maintain the pH of 9,<sup>†</sup> which is optimum for the activity of the enzyme, the buffer system ammonium hydroxide and ammonium chloride is employed<sup>2</sup> in place of the expensive and rather unstable veronal buffer used by King and Armstrong. Buffer solutions of the required pH are prepared easily from solutions of ammonium hydroxide and ammonium chloride. They will keep for long periods of time.

3. Instead of a standard solution of phenol, the amino acid tyrosine is used as a colorimetric standard to estimate the amount of phenol liberated by the action of phosphatase. The standardization of solutions of phenol for colorimetric use offers considerable difficulty. Pure crystalline tyrosine can be obtained readily, and standard solutions are prepared easily by weighing

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<sup>†</sup>It was determined by test that the serum phosphatase has its optimum activity at pH 9.0 to 9.2 in the presence of the ammonium hydroxide-ammonium chloride buffer system.

out the desired amounts of the dry amino acid. The phosphatase units are expressed in terms of the milligrams of tyrosine which give a color equivalent to the phenol liberated.

The new phosphatase method does not present certain difficulties which may be encountered when the method of Bodansky<sup>3</sup> is used, wherein sodium beta-glycerophosphate is employed as the substrate. By the Bodansky method only a small amount of color is normally obtained from the liberated orthophosphate. This is difficult to estimate accurately by ordinary colorimetric procedures. If the Kuttner and Lichtenstein procedure is employed for determining the orthophosphate, it is almost impossible to obtain colorless controls with the reagents.<sup>4</sup>

#### REAGENTS REQUIRED

##### *Buffer Solutions:*

- (1) 0.5 M ammonium chloride: 2.675 Gm. of pure ammonium chloride are dissolved in distilled water to 100 ml. volume, or 26.75 Gm. to 1 liter.
- (2) 0.5 M ammonium hydroxide: 28 ml. of C. P. concentrated ammonium hydroxide are diluted to 1 liter, and the extract strength determined by titration with a standard acid solution using methyl red or methyl orange as an indicator. This should be kept in a stoppered flask to prevent loss of ammonia gas.
- (3) Buffer-substrate mixture: Introduce 1 Gm. of disodium phenyl phosphate, 20 ml. of 0.5 M ammonium chloride, and 20 ml. of 0.5 M ammonium hydroxide into a 100 ml. volumetric flask. Dilute to volume with distilled water and mix.
- (4) 20 per cent trichloroacetic acid: This is approximated by roughly weighing out 20 Gm. of the acid and making up to a volume of 100 ml.
- (5) 2 M sodium hydroxide: 8 Gm. of C. P. sodium hydroxide pellets are weighed out and dissolved in water to 100 ml. volume.
- (6) 1 M sodium hydroxide: The 2 M sodium hydroxide is diluted with an equal volume of water. This is used to neutralize the trichloroacetic acid introduced in the sample in order to precipitate the serum protein. Each batch should be titrated against the 20 per cent trichloroacetic acid in order to determine the amount equivalent to 3 ml. of the acid solution.
- (7) Folin-Ciocalteu reagent:\* This reagent is prepared in the following manner: Dissolve 100 Gm. of sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) and 25 Gm. of sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ) in 700 c.c. of water in a 1,500 c.c. Florence flask. Add 50 c.c. of syrupy (85 per cent) phosphoric acid and 100 c.c. of concentrated hydrochloric acid. Connect the flask with a reflux condenser by means of a cork or rubber stopper wrapped in tin foil. Boil the solution gently for ten hours. At the end of this time, add 150 Gm. of lithium sulfate, 50 c.c. of water, and a few drops of liquid bromine. Boil the mixture without the condenser for about fifteen minutes to remove excess bromine. Cool, dilute to 1 liter, and filter. The finished reagent should have no

\*Portions of the Folin-Ciocalteu reagent sufficient for a day or two may be diluted 1:2 with water, or the reagent may be used in the concentrated form.

greenish tint. It should be kept well protected from dust, because organic materials will gradually produce slight reductions.

- (8) Standard tyrosine solution (5 c.c. = 1 mg. tyrosine): 100 mg. of pure dry tyrosine are accurately weighed and dissolved with approximately 0.1 M hydrochloric acid solution to a volume of 500 ml. in a calibrated volumetric flask. A drop of merthiolate may be added to inhibit the growth of mold.

#### PROCEDURE OF ANALYSIS

*Sample of Blood.*—Five milliliters of blood are drawn from an arm in the usual manner without the addition of an anticoagulant. The blood is allowed to clot and then centrifuged, and if necessary recentrifuged, so that a serum free from suspended cells may be collected. Care must be taken to avoid hemolysis. The separated serum may be stored in a refrigerator for a period as long as a week without any significant change in the phosphatase activity.

*Analysis.*—Two 10 ml. portions of the buffer-substrate mixture are pipetted into rubber-stoppered 15 ml. test tubes. These are placed in a 37.5° C. water thermostat for about five minutes, to bring the substrate to this temperature. Next 0.5 ml. of the test blood serum is added to one of the tubes and mixed with its contents. Both tubes are incubated for exactly one hour at 37° C. The second tube, containing the incubated substrate, is used as a control. After incubation both tubes are removed and placed in an ice bath, or chilled in a refrigerator for at least ten minutes. After chilling, the serum protein should be precipitated with 3 ml. of the 20 per cent trichloroacetic acid. About five minutes after addition of the acid the tubes are centrifuged to separate the precipitated protein. The supernatant liquid is carefully decanted off the protein sediment.

*Control.*—The control is prepared by adding 0.5 ml. of normal serum to the test tube containing the incubated substrate, and chilling. Then the serum protein is precipitated with 3 ml. of 20 per cent trichloroacetic acid. The test tube is centrifuged as above, and the supernatant liquid collected.

*Comparison.*—Into 10 ml. volumetric flasks are pipetted 5 ml. aliquots of the supernatant fluid from the control and test sample. Then 1.0 ml. of the 1.0 M sodium hydroxide is added from a burette to neutralize the acid present. (The exact amount of alkali required may be determined by titrating again a 3 ml. portion of trichloroacetic acid, using phenolphthalein as an indicator. This need be done only once for each fresh batch of alkali and acid.) Next is added 1 ml. of 2 M sodium hydroxide and 0.6 ml. of the concentrated Folin-Ciocalteu reagent or 2 ml. of the reagent diluted to one-third with water. The volumetric flask is filled to the graduation mark with distilled water, and the contents are mixed.

To cover the usual range of phosphatase activity, it is desirable to prepare at least two standard solutions for comparison. Into 50 ml. volumetric flasks are pipetted 2 ml. and 5 ml. of the standard tyrosine solution. To this is added about 20 ml. of water, 5 ml. of 2 M sodium hydroxide, and 3 ml. of concentrated Folin-Ciocalteu reagent or 9 ml. of diluted reagent. It may be necessary at times to use other ranges of tyrosine concentration.

The incubated sample and the control are compared in the colorimeter with the appropriate tyrosine standard.

*Calculation of Phosphatase Activity.*—The phosphatase unit is defined as the amount of enzyme which will liberate a quantity of phenol from 100 ml. of serum in one hour incubation under the described conditions, giving a color equivalent to that produced by 1 mg. of tyrosine. The tyrosine equivalent of the control is subtracted from that of the incubated serum. The difference expresses the tyrosine equivalent of the test sample.

The calculation may be expressed by the following equation:

$$108 \times \frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. Tyrosine in standard} = \text{Tyrosine value.}$$

The tyrosine values of both test and control samples are calculated.

Then: (Tyrosine value in mg. per 100 ml. of test) - (Tyrosine value in mg. per 100 ml. of control) = Phosphatase units.

The factor 108 is derived from a combination of the volume relations of the various steps in the procedure. If the volume of the sample or of the standard solution is altered, then the factor 108 must be altered correspondingly.

*Sera With High Enzyme Content.*—As the hydrolytic products of the phosphate reaction accumulate, they retard the velocity of the reaction.<sup>1</sup> This produces an error in the measurement of the phosphatase units. To minimize this error, sera whose phosphatase content is known to be high should be diluted with normal saline (usually an equal volume) so that the number of units per 100 ml. of the diluted serum will not be in excess of 60.

#### DISCUSSION

*The Phosphatase Unit.\**—Like King and Armstrong,<sup>1</sup> we considered it undesirable to introduce a phosphatase unit that would give values varying too greatly from those already reported. The conditions of the present method were adjusted so as to give sufficient color to the samples and, at the same time, to give a phosphatase unit which in normal subjects would yield phosphatase values of the same magnitude as those reported by King and Armstrong,<sup>1</sup> which are approximately the same as those of Jenner and Kay,<sup>6</sup> and about twice as great as those obtained by the Bodansky method.<sup>3</sup>

*The Time Factor in the Rate of Liberation of Phenol.*—A series of tests showed that, for time periods varying from thirty to ninety minutes, the amount of phenol liberated was very nearly directly proportional to the time. The period of one hour was selected for the present work because in that time an adequate amount of color is produced with about 0.25 ml. of serum, and the tyrosine phosphatase values are nearly equivalent to those of King and Armstrong.

If it is necessary that the incubation period be more or less than the standard time of one hour, then the phosphatase values can be corrected by direct proportion.

\*The relationship between the chromogenic values of tyrosine and phenol was determined. Under similar conditions, the color yielded by 1 mg. of tyrosine was found to be equivalent to 0.524 mg. of phenol.

## ANALYTICAL RESULTS ON THE BLOOD OF NORMAL AND PATHOLOGIC SUBJECTS

The method for phosphatase estimation described has been used in this laboratory for the past two years, and has been evaluated on the blood of normal and pathologic subjects.

TABLE I

COMPARISON OF SERUM PHOSPHATASE VALUES OF NORMAL SUBJECTS BY VARIOUS METHODS

SUBJECT	TYROSINE UNITS	JENNER AND KAY UNITS	BODANSKY UNITS	RATIO
				TYROSINE UNITS BODANSKY UNITS
Tu (1) *	6.4	6.0	3.9	1.6
Tu (2)	5.9		3.0	2.0
Gl	5.0		3.6	1.4
Wei (1)	5.3	5.7	3.5	1.5
Wei (2)	7.0		4.1	1.7
Car	6.0	5.6	3.8	1.6
Du (1)	3.1	4.2	1.7	1.8
Du (2)	6.2		3.5	1.8
Edw	5.7	4.1	2.4	2.4
To	6.2	5.6	4.0	1.6
Ta	9.1	7.2	7.2	1.3
By	6.7	4.2	4.0	1.7
Wi	6.8	5.2	3.2	2.1
Ka	6.4	6.0	4.3	1.5
Co	7.5	5.2	4.3	1.7
Le	7.6	7.8	4.4	1.7
Cas	10.1	8.9	5.4	1.9
Ha	7.2		3.7	1.9
Tom	3.3		1.7	1.9
Fi	6.0		2.4	2.5
Kah	7.2	7.6	3.5	2.0
Number	21	14	21	21
Lowest value	3.1	4.1	1.7	1.3
Highest value	10.1	8.9	7.2	2.5
Arith. mean	6.4	6.0	3.7	1.7

\*The numbered values represent repeated determinations on the same subject.

*Normal Values.*—The phosphatase values obtained by the present method were compared with those obtained by the method of Bodansky and that of Jenner and Kay. The subjects were young adults, both male and female graduate students. The results are recorded in Table I. Twenty-one estimations showed a range of between 3.1 to 10.1 units, with an average of 6.4. In these subjects the Bodansky method gave a range of between 1.7 and 7.2 units, with an average of 3.7. The average ratio between tyrosine units and Bodansky units was 1.7. In 14 subjects the Jenner and Kay method yielded a range of 4.1 to 8.9 units, with an average of 6.0. This demonstrates that the value of the present method agrees closely with the values of King and Armstrong and Jenner and Kay. The values marked (1) and (2) on the same subject represent repeated determinations at varying intervals of time.

*Values in Pathologic Conditions.*—The many recent studies on phosphatase have shown that an increase in the phosphatase activity of blood serum occurs in certain diseases of the bone and in certain types of jaundice. Increased phosphatase values have been found in osteitis deformans (Paget's disease of bone),<sup>4, 7-11</sup> in osteitis fibrosa cystica,<sup>9, 10, 12</sup> and in cancer with osteoplastic metastases.<sup>4, 7, 8, 13-16</sup> In the osteoplastic type of bone sarcoma the plasma phosphatase was found to be particularly high, whereas it remained about

TABLE II  
SERUM PHOSPHATE VALUES IN DISEASES WITH BONE INVOLVEMENT

SUBJECT	TYROSINE UNITS	BODANSKY UNITS	RATIO	DIAGNOSIS
<i>A. Paget's Disease (Osteitis deformans)</i>				
T. A. (1)	97.3	38.4	2.5	
T. A. (2)	98.9	38.3	2.6	
L. L. S.	35.5	20.9	1.7	
L. G. L. (1)	45.1	16.9	2.7	
L. G. L. (2)	48.0	16.4	2.9	
L. H.	30.0			
C. T.	20.9			
K. N.	19.3			
M. G.	6.6	2.4	2.7	
F. O.	26.0			
S. H.	18.6			
A. B.	23.0	10.3	2.2	
Number	12	7	7	
Lowest value	6.6	2.4	1.7	
Highest value	98.9	38.4	2.9	
Arith. mean	40.8	20.5	2.5	
<i>B. Hyperparathyroidism (Osteitis fibrosa cystica)</i>				
N. G.	22.3	9.2	2.4	
E. W.	21.4	9.1	2.4	
Arith. mean	21.9	9.2	2.4	
<i>C. Various Diseases Demonstrating Involvement of Bone and Abnormalities of Calcium Metabolism</i>				
L. D.	5.2	1.5	3.7	Desmoud tumor of bowel
J. S.	6.9	4.2	1.7	Multiple bone tumor
M. D.	6.5	4.3	1.5	Giant tumor of tibia
K. R.	3.4	3.0	1.1	Osteomyelitis of spine
M. A.	6.1	2.3	2.7	Osteoporosis
A. C.	12.7			Osteoporosis
C. F. S.	11.3			Osteoporosis
T. K.	9.7			Hodgkin's disease of bone
G. H.	6.8			Ankylosis of hips
R. O.	23.7			Intracranial lesions
D. S. (1)	9.9	4.8	2.0	Osteoporosis
D. S. (2)	11.7	4.9	2.4	Nephrolithiasis
C. O.	11.2	4.8	2.3	Nephrolithiasis
L. S.	41.5	14.6	2.8	Nephrolithiasis
				Plasma cell myeloma
Number	14	9	9	
Lowest value	3.4	1.5	1.1	
Highest value	41.5	14.6	3.7	
Arith. mean	11.9	4.9	2.2	

normal in the osteolytic type of bone sarcoma, multiple myeloma, endothelial myeloma, chondroma, and chondrosarcoma.

The serum phosphatase also has been found to be high in obstructive jaundice, acute hepatitis, and hepatic cirrhosis.<sup>17-21</sup> It remains normal in catarrhal, infective, and hemolytic jaundice.

In the present study, samples were taken from 10 subjects with conditions diagnosed as Paget's disease of the bones, from 2 patients with hyperparathyroidism, and from 13 patients with various diseases of bone and abnormalities of calcium metabolism. The results are given in Table II. In Table II A are tabulated the cases of Paget's disease. The phosphatase values found in



TABLE III

## SERUM PHOSPHATASE VALUES IN DISEASES WITHOUT BONE OR CALCIUM INVOLVEMENT

SUBJECT	TYROSINE UNITS	BODANSKY UNITS	RATIO	DIAGNOSIS
<i>A. Diseases Accompanied by Icterus</i>				
A. M. R.	56.1			Portal cirrhosis
W. G. M.	58.3	37.2	1.6	Obstructive jaundice
S. R.	48.3	26.8	1.8	Cirrhosis of liver
K. P.	39.8			Cirrhosis of liver
H. C.	11.5	5.5	2.1	Carcinoma of liver
J. L.	5.6	3.1	1.8	Hypertrophic biliary cirrhosis
				Acute hemolytic icterus
Number	6	4	4	
Lowest value	5.6	3.1	1.6	
Highest value	58.3	37.2	2.1	
Arith. mean	28.3	18.2	1.8	
<i>B. Various Diseases Without Bone Involvement or Abnormalities of Calcium Metabolism</i>				
N. H.	11.2	4.3	2.6	Undulant fever
S. R.	3.1	2.0	1.6	Duodenal ulcer
M. L.	5.2	5.1	1.0	Senile purpura
A. J. (1)	8.7	4.6	1.9	Paroxysmal nocturnal hemoglobinuria
A. J. (2)	9.5	3.9	2.4	
C. K.	4.3	3.6	1.2	Polycythemia hypertonica
P. P. (1)	6.5	4.4	1.5	Silicosis
P. P. (2)	6.9	5.6	1.2	
S. T.	10.4	7.4	1.4	Myotonia congenita
A. F.	5.9	3.3	1.8	Diabetes mellitus
M. I.	6.9	3.1	2.2	Hyperthyroidism
E. P.	8.9	6.3	1.4	Erythromelalgia
C. O. (1)	4.1	--	--	
C. O. (2)	5.7	1.3	4.4	Anxiety neurosis
G. B.	8.2	4.3	1.9	Chronic glomerular nephritis
A. S. P.	29.6	22.2	1.4	Syphilis
Number	16	15	15	
Lowest value	3.1	2.0	1.0	
Highest value	29.6	22.2	4.4	
Arith. mean	8.45	5.4	1.6	

this condition were high in every instance except one. The ratio of tyrosine units to Bodansky units in this series of cases averaged 2.5.

Table II B shows the results obtained in 2 patients with hyperparathyroidism, in both of whom the values were about three times the normal mean. Results obtained in the series demonstrating abnormalities of calcium metabolism and diseases of bone are given in Table II C. These results are in agreement with those obtained in the work already cited. Only in one case of plasma cell myeloma and in another with decalcification of unknown etiology was there a marked increase in the serum phosphatase. The 2 persons with nephrolithiasis listed in the table are of interest because of the singularly close agreement between the phosphatase values of the two.

Table III A shows the phosphatase values of serum samples obtained from patients with icterus. These, too, are in agreement with the observations recorded in the literature. In cirrhosis and obstructive jaundice there is a great increase in the phosphatase value of the serum. In acute hemolytic icterus the plasma phosphatase is normal.

In Table III B are recorded the phosphatase values for a number of patients with various diseases without bone involvement or abnormalities of calcium metabolism. With the exception of high values in a case of syphilis, the results obtained fell fairly well within the range of values found in the groups of normal controls. The average of the ratios of tyrosine units to Bodansky units in this group of patients is nearly the same as that found in the group of normal subjects.

#### SUMMARY

1. A revision of the serum phosphatase method of King and Armstrong, in which disodium phenyl phosphate is used as the substrate, is presented.
2. The revised method eliminates certain sources of inaccuracy. It is simpler, and is especially well suited for clinical use.
3. The phosphatase values obtained by the new method agree in general with those previously obtained by other methods.

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# A COMPARATIVE STUDY OF BLOOD AND SPINAL FLUID BY THE KAHN, KLINE, AND LAUGHLEN TESTS\*

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SINCE Laughlen<sup>1</sup> described a new flocculation test for syphilis in 1935, attempts have been made by many workers to determine the reliability of this test as compared with other well-known methods, namely, Wassermann, Kahn, and Kline.

In his first report Laughlen<sup>1</sup> found that his test gave 98 per cent agreement with the Wassermann test, and 99 per cent agreement with the Kahn test, using a small number of specimens—400 blood and 20 spinal fluid samples. Later he<sup>2</sup> studied over 5,000 ward specimens, comparing the Laughlen with the Wassermann, Hinton, and Kahn methods. On the first 2,000 specimens examined, agreement with the Wassermann test was 97 per cent, and with the Kahn 98 per cent. The complete report showed an agreement with the Wassermann of over 98 per cent, and with the Kahn 99.4 per cent. Laughlen's original work has been confirmed by several groups of workers. Robinson and Stroud<sup>3</sup> performed the Laughlen, Wassermann, and Kahn tests on 1,000 sera. They found 93 per cent agreement between the Laughlen and Wassermann tests, and 97 per cent agreement between the Laughlen and Kahn tests. Muether and Greutter,<sup>4</sup> after performing 1,000 tests on blood say, "The Laughlen test is slightly less accurate than the Kahn test and compares fairly well with the Kline test." Craig and Callaway<sup>5</sup> found on 1,000 patients that the Laughlen test agreed with the Kahn and Wassermann tests in 91.8 per cent of their cases. With the Wassermann test alone there was agreement in 91.8 per cent. With the Kahn test alone 99.8 per cent agreement was obtained. Breazeale, Greene, and Harding<sup>6</sup> performed the Kahn, Eagle, Ide, Kline, and Laughlen tests on 1,000 sera. A comparison of the results showed that the agreement between the tests varied from 98.4 per cent to 99.5 per cent. Over 10 per cent of their sera were tested by three other laboratories using the Wassermann tests. There was agreement in 94.5 per cent of the cases. Usher's<sup>7</sup> work with the Laughlen test led him to conclude that it was less specific than the Wassermann and Eagle tests, and when the sensitivity of the reagent was reduced enough to eliminate false positive reactions, it failed in a large number of cases to detect positive sera which gave a positive reaction to the Eagle flocculation test. Flood and Mayer<sup>8</sup> found that the Laughlen and Ide tests were in agreement with the standard tests—Kline, Kahn, and Kolmer—in about 81.95 per cent of 1,025 cases and on 67.93 per cent of 711 specimens of

\*From the Laboratory of the Eastern State Hospital, Medical Lake.  
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known syphilities. These various studies caused an editorial<sup>9</sup> and a current comment<sup>10</sup> warning against the use of the Laughlen test by untrained people.

The present study was undertaken to compare the Laughlen test with the Kahn and Kline tests. The three tests were used routinely on all sera and spinal fluids in this report. Over 20 per cent of the sera and spinal fluids were tested by another laboratory,\* using the Wassermann test. There was agreement in over 99 per cent of the cases. Patients used in this study were principally those admitted to this hospital. To a small extent the patients were those under treatment in the Syphilis Clinic and were known syphilities.

It was decided to perform the Kahn, Kline, and Laughlen tests on at least 1,500 blood samples and 1,000 spinal fluid specimens. The Kahn and Kline tests are used routinely in this laboratory. A group of 150 bloods and 150 spinal fluids were performed by the Laughlen test as a preliminary step in order to familiarize myself with the test before running the larger series. Positive and negative controls were run throughout this study. All tests were done personally. The sera in these tests were inactivated at 56° C. for thirty minutes.

The Kahn precipitation test was performed as described by Kahn.<sup>11</sup> The antigen, a cholesterolized one, was purchased from Sharp and Dohme Co. The Kline diagnostic tests were done as described by Kline.<sup>12</sup> The antigen and reagents for its use were purchased from La Motte Co., Baltimore. All the Laughlen tests were run as described by Laughlen.<sup>1</sup> The Laughlen antigen was prepared from a cholesterolized Kahn antigen, using scarlach R dye. Great care was used in preparation of the Laughlen antigen following Laughlen's<sup>1</sup> directions. Good results were usually obtained after a little practice.

A total of 1,547 serum tests and 1,002 spinal fluid tests were done in this series. Table I shows the number of tests performed for each method used. The Wassermann tests shown in all the tables are presented for comparison, indicating that over 20 per cent of the tests were checked in a separate laboratory. All tests are in close agreement with those shown in Tables II and III.

TABLE I  
TOTAL NUMBER OF TESTS

	WASSERMANN	KAHN	KLINE	LAUGHLIN
Blood tests	392	1,547	1,547	1,547
Spinal fluid tests	207	1,002	1,002	1,002

TABLE II  
COMPARISON FOR SPECIFICITY

		WASSERMANN	KAHN	KLINE	LAUGHLIN
Blood tests	Positive tests	163	365	364	352
	Negative tests	229	1,182	1,183	1,195
Spinal fluid tests	Positive tests	74	251	254	245
	Negative tests	133	751	748	757

\*Washington State Department of Health, Seattle.

TABLE III  
COMPARISON FOR SPECIFICITY

		WASSERMANN	KAHN	KLINE	LAUGHLIN
Blood tests	Positive tests	163	164	162	161
	Negative tests	229	228	230	231
Spinal fluid tests	Positive tests	74	75	73	73
	Negative tests	133	132	134	134

#### DISCUSSION

If it were possible to obtain 100 per cent results in all the methods used, each method should give 367 positive sera, 1,180 negative sera, 253 positive spinal fluids, and 749 negative spinal fluids. It was assumed that when a majority of the tests performed on a sample of blood or spinal fluid gave the same result that result was the correct one. Any test not agreeing with the majority of the tests run on a given sample was considered wrong unless proved otherwise by repeated tests on samples from the same patient.

Of the methods used in testing the sera, the Kahn test showed only 4 false tests, the Kline showed 5 false tests, and the Laughlen gave 17 false tests. In the spinal fluid examinations the Kline gave the best results, with just one false test. The Kahn was next with three errors. The Laughlen test was last with 10 tests that were wrong. In both the sera and the spinal fluid examinations the Kahn and the Kline were over 99 per cent accurate in regard to specificity. The Laughlen was slightly below, with an accuracy of more than 98 per cent.

The agreement between the Laughlen, Kahn, and Kline tests obtained in this work is higher than that reported by most authors. This may be explained by the following reasons:

1. Preliminary experience in performing the tests was secured by testing a group of one hundred or more before applying it in the present comparative study.

2. Well-known antigens, properly standardized, were used in all the work.

3. Great care was used in preparing the Laughlen antigen. In doing this I found that the total time in doing the Laughlen test was nearly as much as was needed for the Kahn and Kline tests.

4. All the individuals that had had syphilis had the disease for years. There were no cases of primary or secondary syphilis. Many of these patients had had no treatment. Some of them had been insufficiently treated, having received only a few injections of heavy metals. A few of them were under treatment for years before commitment, and still gave a strongly positive test for syphilis.

#### CONCLUSIONS

The Laughlen test is slightly less specific than the Kahn and Kline.

To secure accurate results the time needed to perform the test is nearly the same as that needed for the Kahn and Kline tests.

Inexperienced workers should not use the Laughlen test for the diagnosis of syphilis.

I am indebted to Dr. Harry N. Roback for his kind advice, criticism, and helpful suggestions throughout this study.

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THE LANGE TEST

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III. THE USE OF THE PHOTELOMETER IN MAKING LANGE TEST READINGS

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ALL clinical laboratories visited by us rely on direct visual observation in deciding the colors obtained in the Lange<sup>1</sup> test. Although the experienced technician is able to decide very accurately whether the color is a 0, 1, 2, 3, 4, or 5, the less experienced operator is not always so certain of his readings. Hence, it would appear to be desirable to try to develop a convenient and accurate mechanical method for measuring the colors. This study summarizes results obtained using a photelometer for making Lange test readings.

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THE PHOTELOMETER

A standard Cenco-Sheard-Sanford Photelometer, described in detail in Bulletin No. 104 of the Central Scientific Co., was used. A special carrier built to hold eleven 16 mm. x 55 mm. pyrex test tubes was used in place of the rectangular absorption cell provided with the instrument. This carrier was mounted on a track in such a manner that it could be easily moved back and forth and could be locked in position so that the beam of light would pass through the center of the particular test tube being examined.

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EXPERIMENTAL

Using sols prepared by the nuclear formaldehyde method of Glasoe and Sorum<sup>2</sup> and the sodium citrate method of Borowskaja,<sup>3</sup> the authors ran Lange tests on available paretic and syphilitic spinal fluids. The tests were read

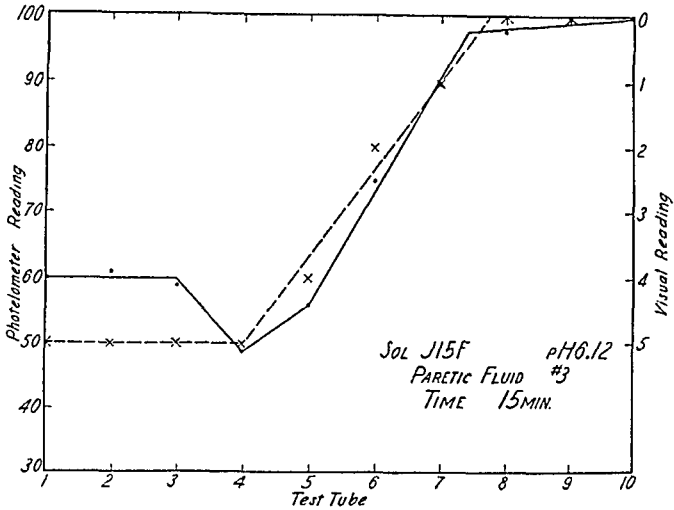


Fig. 1.—Lange test reading with paretic fluid.

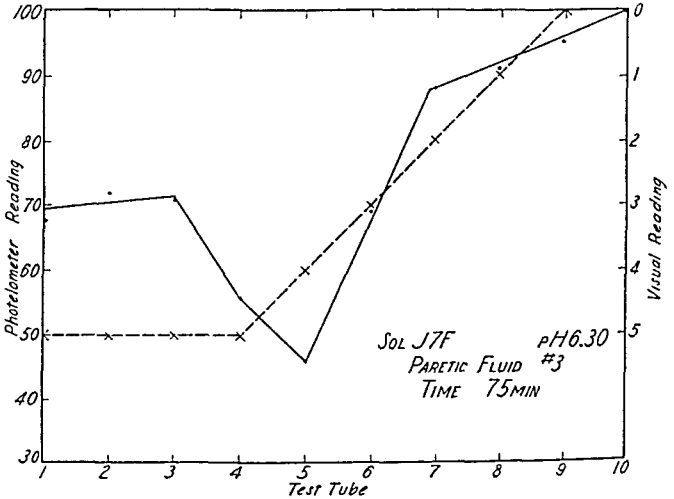


Fig. 2.—Lange test reading with paretic fluid.

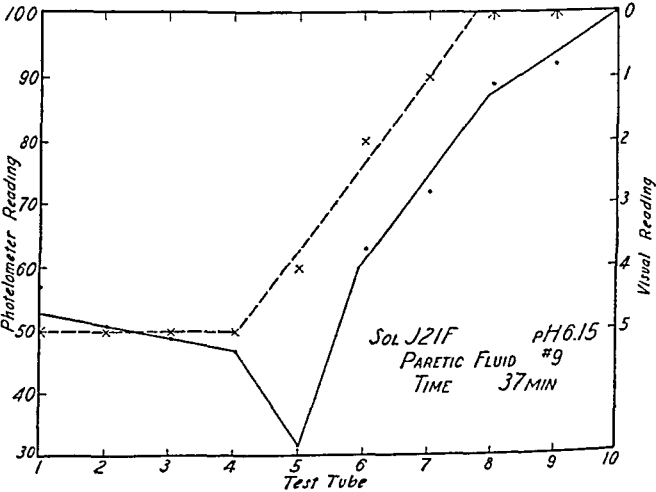


Fig. 3.—Lange test reading with paretic fluid.

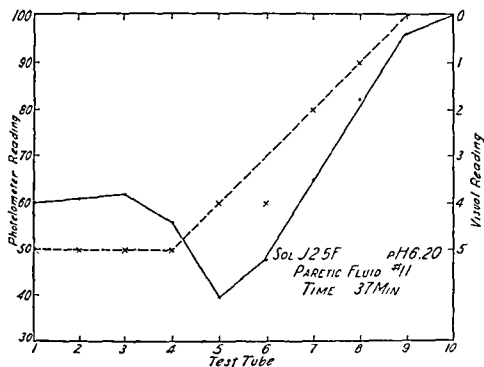


Fig. 4.—Lange test reading with paretic fluid.

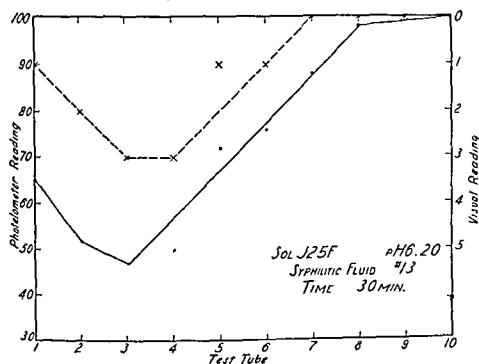


Fig. 5.—Lange test reading with syphilitic fluid.

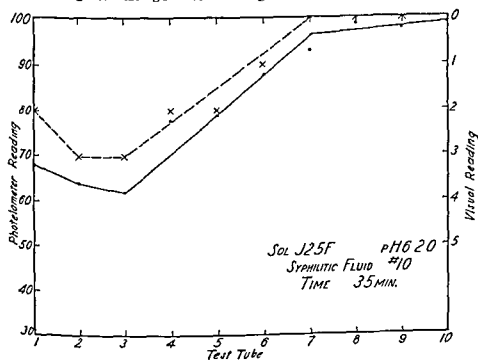


Fig. 6.—Lange test reading with syphilitic fluid.



with the photelometer, and the corresponding visual readings were taken. The results are shown in the form of graphs (Figs. 1 to 7). The photelometer readings are represented by dots, and the curve is drawn as a solid line. The visual readings are represented by crosses, and the curve drawn with a broken line. The time given is the time elapsed between the addition of the gold sol and the photelometric reading. The visual readings were taken one to one and one-half hours after mixing.

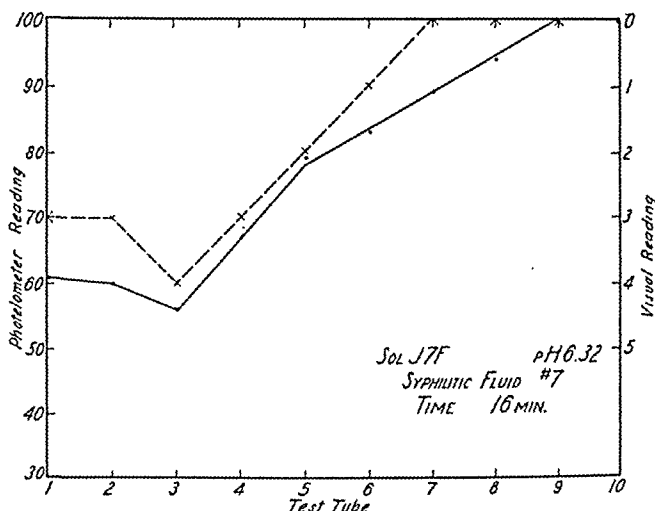


Fig. 7.—Lange test reading with syphilitic fluid.

To determine the influence of time on the photelometric readings, the first tests made with the photelometer were read at two-minute intervals for the first twenty minutes, then at longer intervals up to five hours. The characteristics of the curves did not change after the first fifteen minutes. Therefore, most of the readings were taken fifteen to thirty minutes after mixing.

#### CONCLUSIONS

The results obtained in these experiments indicate that the photelometer provides a very sensitive and accurate method for making Lange test readings. Its use enables the less experienced operator to make fine differentiations of color that are not possible with direct visual readings. In particular it makes possible the detection of even slight differences in the color of gold sols; as such it is an invaluable tool in Lange test research. However, in practical clinical work, where the general form of the curve and the relative colors of the solutions are more important than the actual colors, the use of the photelometer appears to have no advantage over the visual method.

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## A TRIPLE SUGAR-FERROUS SULFATE MEDIUM FOR USE IN IDENTIFICATION OF ENTERIC ORGANISMS\*

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THE need for a simple practical medium to be used in teaching and public health laboratories for the differentiation of the colon-typhoid-dysentery group is evidenced by the numerous culture methods described for the isolation of this group of organisms. A simple medium which would afford a maximum amount of information in a minimum time is most desirable. Several examples of such media have been described. Russell,<sup>1</sup> for example, devised a simple medium containing nutrient agar, lactose, glucose, and an indicator, for differentiation between the lactose-fermenting and nonlactose-fermenting gram-negative bacilli. Krumwiede and Kohn<sup>2</sup> later modified this medium by introducing a third sugar, saccharose, in order to obtain more information in a single medium. Kligler<sup>3</sup> described a simple medium containing lead acetate for the detection of hydrogen sulfide which could be successfully combined<sup>4</sup> with the Russell double sugar medium. The medium of Kligler did not receive widespread use in public health laboratories; subsequently the method of its preparation was simplified by Bailey and Lacy,<sup>5</sup> who determined the optimum temperature for mixing the ingredients of the medium, and the best method for its sterilization.

The use of iron salts for the detection of hydrogen sulfide has been recommended by a large number of investigators, including Treece,<sup>6</sup> Wilson,<sup>7</sup> Beckwith and Moser,<sup>8</sup> Levine, Vaughn, Epstein, and Anderson,<sup>9</sup> Zobell and Feltham,<sup>10</sup> and others. Bismuth has been recommended as an indicator by Pacheco and Toledo Mello,<sup>11</sup> and more recently by Hunter and Crecelius.<sup>12</sup>

In the selection of a suitable indicator system for detection of hydrogen sulfide, one must take into consideration several factors, including toxicity, sensitivity, and distinctness of color change. Although it is not clear that evolution of hydrogen sulfide from media containing proteins is necessarily evidence of the reduction of sulfur-containing compounds, one must also consider the available source of sulfur in selecting a suitable indicator system. Bacteria which produce hydrogen sulfide seem to do so more freely if cystine or other sulfur-containing compounds (taurine, sodium sulfite, sodium thiosulfate, thiourea) are added to the medium.

Several experiments were performed to determine the relative sensitivity of ferrous sulfate, ferric ammonium citrate, and bismuth citrate, using several strains of freshly isolated organisms, and a large number of strains of the

\*From the Laboratories of the St. Louis Health Division.  
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TABLE I

## HYDROGEN SULFIDE REACTIONS IN MEDIA CONTAINING DIFFERENT INDICATORS

NAME OF ORGANISM	NUMBER OF STRAINS	MEDIUM I				MEDIUM II				MEDIUM III			
		FERROUS SULFATE AND SODIUM THIOSULFATE				FERRIC AMMONIUM CITRATE AND SODIUM THIOSULFATE				BISMUTH LIQUOR*			
		18 HR.	24 HR.	36 HR.	48 HR.	18 HR.	24 HR.	36 HR.	48 HR.	18 HR.	24 HR.	36 HR.	48 HR.
<i>E. typhosa</i>	12	12	0	0	0	11	1	0	0	10	2	0	0
<i>E. typhosa</i>	30†	28	2	0	0	26	3	1	0	25	4	1	0
<i>S. paratyphi</i>	9	0	0	0	0	0	0	0	0	1	2	0	0
<i>S. schottmülleri</i>	4	4	0	0	0	4	0	0	0	4	0	0	0
<i>S. hirschfeldii</i>	1	1	0	0	0	1	0	0	0	1	0	0	0
<i>Shig. dysenteriae</i>	3	0	0	0	0	0	0	0	0	1	1	0	0
<i>Shig. paradysenteriae</i> (Flexner)	5	0	0	0	0	0	0	0	0	0	2	1	0
<i>Shig. paradysenteriae</i> (Hiss-Russell)	2	0	0	0	0	0	0	0	0	0	0	1	0
<i>S. suipestifer</i>	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. aertrycke</i>	9	8	1	0	0	9	0	0	0	9	0	0	0
<i>S. morgani</i>	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>S. anatum</i>	3	3	0	0	0	2	1	0	0	3	0	0	0

\*The bismuth liquor consisted of a 3 per cent stock solution of bismuth citrate (Merck U.S.P. VIII), prepared as suggested by Hunter and Crecelius.<sup>12</sup>

†Freshly isolated strains of *E. typhosa*.

Note.—Figures refer to number of strains showing hydrogen sulfide production after indicated number of hours' incubation at 37° C.

typhoid-dysentery group of organisms kindly supplied by Dr. McDonald Fulton, of the St. Louis University School of Medicine.

In the first set of experiments different hydrogen sulfide indicator systems\* were added to a beef infusion agar base containing bacto-peptone:

	Medium I	
Ferrous sulfate		0.02 per cent
Sodium thiosulfate		0.015 per cent
	Medium II	
Ferric ammonium citrate		0.05 per cent
Sodium thiosulfate		0.03 per cent
	Medium III	
"Bismuth liquor"		0.5 per cent
(3.0 per cent bismuth citrate)		

Eighty (80) strains of organisms were tested in these media in order to determine the relative sensitivity of the respective indicators. In order to be assured of uniformity approximately the same amount of inoculum from eighteen-hour broth cultures was used in seeding the respective media. The cultures were incubated at 37° C. and examined after eighteen, twenty-four, thirty-six, and forty-eight hours. As seen in Table I, medium I contained the most effective hydrogen sulfide indicator system. The presence of hydrogen sulfide could be detected in less than eighteen hours. Only 3 of 59 strains producing hydrogen sulfide required more than eighteen hours' incubation at 37° C. The ferric ammonium citrate was somewhat less sensitive than the ferrous sulfate, while the bismuth citrate was considerably more sensitive. Three strains each of *Salmonella paratyphi*, and *Shigella paradysenteriae*

\*The concentration of indicators used was established in a preliminary experiment.

(Flexner), two strains of *Shigella dysenteriae*, and one Hiss-Russell strain showed hydrogen sulfide production, as demonstrated by use of the bismuth citrate indicator (medium III). This indicator is perhaps too sensitive, since these organisms are generally considered to be negative hydrogen sulfide formers, and the differential value of hydrogen sulfide production in routine diagnostic work would no longer be valid. The use of such a sensitive indicator would necessitate revision of the generally accepted classification of the enteric organisms, or the presence or absence of hydrogen sulfide should be recorded according to the medium or indicator used.

Another set of experiments was carried out to determine the effect of changes in hydrogen-ion concentration on the effectiveness of the respective indicators. The media used in the previous experiment were prepared in buffered solutions ranging from pH 5.0 to pH 9.0. An eighteen-hour broth culture of a freshly isolated strain of *E. typhosa* was used as the test organism. The results, summarized in Table II, show that changes in the hydrogen-ion concentration had no effect on the sensitivity of the bismuth citrate for detection of hydrogen sulfide, confirming the observation of Hunter and Crecelius.<sup>12</sup> The ferric ammonium citrate, however, apparently reacts very slowly with the hydrogen sulfide to form iron sulfide. This indicator failed to detect the hydrogen sulfide in an acid medium (pH 5.0) even after incubation for fourteen days. The ferrous sulfate, on the other hand, was only slightly affected by changes in hydrogen-ion concentration of the menstruum. Essentially the same results were obtained when this experiment was repeated at a later date.

TABLE II  
EFFECT OF HYDROGEN-ION CONCENTRATION ON SENSITIVITY OF IRON AND BISMUTH SALTS TO HYDROGEN SULFIDE

pH	FERROUS SULFATE AND SODIUM THIOSULFATE	FERRIC AMMONIUM CITRATE AND SODIUM THIOSULFATE	BISMUTH CITRATE
5.0		—	+++
5.5	±	±	+++
6.0	+	+	+++
6.5	++	++	+++
7.0	++	++	+++
7.5	+++	+++	+++
8.0	+++	++	+++
8.5	+++	+	+++
9.0	++	+	+++

— = No hydrogen sulfide detectable after incubation at 37° C. for seven days.

± = Slight amount of hydrogen sulfide formed.

+= Moderate hydrogen sulfide production.

++ = Abundant hydrogen sulfide production.

+++ = Abundant hydrogen sulfide production within eighteen hours after incubation at 37° C.

In another set of experiments bacto-tryptone,\* which is especially adapted for use in the elaboration of indol by bacteria, was found to be considerably more suitable than bacto-peptone from a viewpoint of available sulfur content, growth-promoting properties, and uniformity of results (Table III).

\*Prepared by the Difco Laboratories, Inc., Detroit, Mich.

TABLE III

## EFFECT OF PEPTONE AND TRYPTONE ON HYDROGEN SULFIDE PRODUCTION

NAME OF ORGANISM	NUMBER OF STRAINS	BACTO-PEPTONE				BACTO-TRYPTONE			
		18	24	36	48	18	24	36	48
<i>E. typhosa</i>	12	3	1	8	0	8	1	3	0
<i>E. typhosa</i>	6*	2	1	2	1	5	1	0	0
<i>S. paratyphi</i>	6	0	0	0	0	0	0	0	0
<i>S. schottmüller</i>	4	4	0	0	0	4	0	0	0
<i>Shig. dysenteriae</i>	3	0	0	0	0	0	0	0	0
<i>Shig. paradysenteriae</i> (Flexner)	5	0	0	0	0	0	0	0	0
<i>S. aertrycke</i>	6	4	2	0	0	6	0	0	0

\*Freshly isolated strains of *E. typhosa*.

Note.—Figures equal number of strains showing H<sub>2</sub>S production after indicated number of hours' incubation at 37° C.

The ferrous sulfate-sodium thiosulfate indicator system was added to two batches of medium containing bacto-peptone and bacto-tryptone, respectively. Several strains (42) of organisms of the typhoid-dysentery group were tested. Cultures were examined after incubation at 37° C. for eighteen, twenty-four, thirty-six, and forty-eight hours. Hydrogen sulfide could be detected in less than eighteen hours in 23 of 28 hydrogen sulfide formers when grown in the medium containing bacto-tryptone, while only 13 strains showed hydrogen sulfide in less than eighteen hours when grown in the bacto-peptone medium.

It has recently come to our attention that the Difco Laboratories, Inc. have prepared a dehydrated medium, identified as bacto-Kligler iron agar, designed to replace bacto-Kligler lead acetate agar. This medium is very similar to the one described here. Ferric ammonium citrate is used as the hydrogen sulfide indicator instead of ferrous sulfate. The Difco medium likewise permits differentiation of the gram-negative rods both on the basis of their ability to ferment dextrose or lactose and on their ability to produce hydrogen sulfide, and has given comparable results with our own medium.

The hydrogen-sulfide indicator system described in the present report has been successfully combined with Krumwiede's triple sugar medium. The following method of preparation proved satisfactory:

## Agar Base

Beef extract	5.0 Gm.
Bacto-tryptone	20.0 Gm.
Sodium chloride	5.0 Gm.
Agar	10.0 Gm.
Water	1,000 c.c.
Adjust to pH 7.4-7.6 and sterilize at 15 pounds pressure for fifteen minutes.	

## Modified Triple Sugar Medium

To the melted base cooled to 50° C. add	
Lactose	1.0 per cent
Saccharose	1.0 per cent
Glucose	0.1 per cent
Ferrous Sulfate	0.02 per cent
Sodium thiosulfate	0.015 per cent
Bromthymol blue (1.6 per cent alcoholic solution)	0.10 per cent
Sterilize at 5 pounds pressure for twenty minutes.	

When only small amounts of medium are used, it is desirable to add the carbohydrates (sterilized by filtration) to the melted base which has been cooled to 50° C. Appropriate amounts of 2 per cent solutions of ferrous sulfate and sodium thiosulfate (previously sterilized at 5 pounds pressure for twenty minutes) are then added to the medium, together with the bromthymol blue indicator. However, for routine use in public health diagnostic laboratories the ingredients can be mixed in bulk at a temperature of 50° C., dispensed in small homeopathic vials, and sterilized by heating in the autoclave at 5 pounds pressure for twenty minutes.

Only 1.0 per cent agar is used in order to obtain a semisolid medium which permits the motile organism to grow away from the line of inoculation, giving a brushlike appearance which can be used as an additional means of differentiation.

#### SUMMARY

1. A modification of the Krumwiede triple sugar medium is described for use in the differentiation of the colon-typhoid-dysentery group of organisms.

2. Tryptone (bacto) is more suitable than other peptones from a viewpoint of available sulfur content, growth-promoting properties, and uniformity of results.

3. The ferrous sulfate-sodium thiosulfate indicator system is more sensitive than the ferric ammonium citrate-sodium thiosulfate system for the detection of hydrogen sulfide.

4. The sensitivity of bismuth citrate is not affected by changes in pH, ferrous sulfate is only slightly affected, and ferric ammonium citrate reacts very poorly in an acid menstruum.

5. The use of bismuth citrate, which is an extremely sensitive indicator, would necessitate revision of the generally accepted classification of the enteric organisms.

6. In recording the presence or absence of hydrogen sulfide in cultures the medium and indicator system used should be stated.

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## A BEDSIDE TEST FOR SULFAPYRIDINE\*

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IT IS desirable to know the concentration of sulfapyridine in the blood when treating pneumococcic pneumonias and their complications. That the bactericidal effect of sulfapyridine is directly proportional to its concentration has been amply shown. The concentration of sulfapyridine in the blood is determined by the rate of absorption, the rate of excretion, and the rate of conversion. Absorption, excretion, and conversion vary independently and are influenced by factors we do not fully understand. In adults with a uniform dosage of 1 Gm. after an initial larger dose, concentrations varying from 1 to 10 mg. per cent (1:100,000 to 1:10,000) were obtained.

Concentrations too high do not occur with the usual dosage, but the concentration may be too low for bactericidal or bacteriostatic action. Unless the parts per thousand in the blood or milligrams per hundred cubic centimeters are known, clinical failure cannot be ascribed either to inefficiency or to inadequate concentration of the drug. At times, even high concentrations for some resistant strains were insufficient to achieve early bacteriostasis. In our cases treated successfully with sulfapyridine, 4 to 6 mg. per 100 c.c. were usually present in the blood. In some cases of pneumococcic meningitis 10 mg. per 100 c.c. of cerebrospinal fluid were required to effect a cure. The concentration in the cerebrospinal fluid is usually two-thirds that in the blood. When the concentration is too low, larger and more frequent oral doses or parenteral administration may be employed.

Ready extraction of sulfapyridine from the blood with ether suggested a simplification of the Marshall test.<sup>1</sup> Our modification may be performed away from a laboratory by the physician or by the nurse at the bedside, and with apparatus within the means of the smallest hospital. The test, with a simultaneous duplicate analysis, may be carried out in ten minutes.

The following reagents and apparatus are required for the determination of sulfapyridine by our method.

### Reagents:

- (1) Ether
- (2) 15 per cent trichloroacetic acid solution
- (3) 0.1 per cent solution of sodium nitrite

\*From the Medical Service, Harlem Hospital, Department of Hospitals, New York City, and the Littauer Pneumonia Research Fund, New York University College of Medicine.  
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- (4) 1 per cent solution of urea
- (5) A solution of  $\alpha$ -dimethylnaphthylamine containing 1 c.c. in 250 c.c. of 95 per cent ethyl alcohol. (This reagent should be kept in a dark dropping bottle.)

#### Apparatus:

- (1) 2 c.c. Luer syringe and needle
- (2) 1 Test tube of 20 c.c. capacity with round bottom, stoppered with cork and marked at 1 c.c. and 6 c.c.
- 1 Centrifuge tube with long taper, graduated to 15 c.c. in 0.1 c.c. divisions, fitted with rubber stopper
- (3) 5 Dropping bottles to contain the reagents listed above
- (4) A comparator block 2.5 inches by 4.5 inches, with 4 openings to take test tubes 100 by 12 mm., and color standards.\*

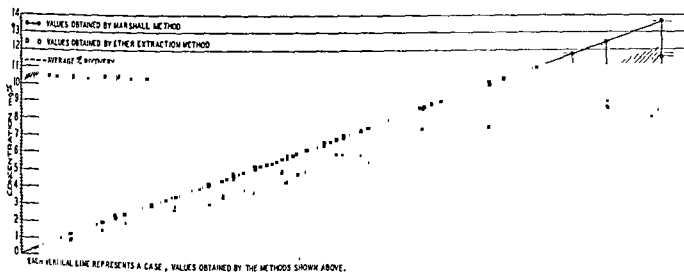


Chart 1.—Comparative values for sulfapyridine in 45 patients by the Marshall method and by the ether extraction method.

#### METHOD

Into a Luer syringe draw approximately 1.5 c.c. of venous blood. Invert the syringe with the needle still attached and deliver the blood, drop by drop, into the round-bottomed test tube, to the 1 c.c. mark. From the other dropping bottle add ether to the 6 c.c. mark. Insert the stopper and shake vigorously for two minutes. The fluids will rapidly separate into two layers, with the ethereal extract of sulfapyridine in the upper layer. Slowly release the stopper. Cautiously decant the ethereal extract into the centrifuge tube to the 0.5 c.c. mark and set the extraction tube aside for duplicate tests.

By means of a dropping bottle add 15 per cent trichloroacetic acid solution to the 5 c.c. mark, place the rubber stopper over the mouth of the tube, and shake vigorously for ten to twenty seconds. Add 0.5 c.c. of 0.1 per cent sodium nitrite solution (7 to 8 drops from a dropping bottle), bringing the solution to the 5.5 c.c. mark. Again shake vigorously for twenty seconds, and add 0.5 c.c. of 1 per cent urea solution dropwise from a dropping bottle. Finally, add  $\alpha$ -dimethylnaphthylamine to the 8.5 c.c. mark from its dropping bottle, close the tube with the rubber stopper, and invert once or twice. The white

\*Reading is facilitated when the width of the color band is increased by placing tubes filled with water behind the standard and the test.



opalescence is soon replaced by a purplish red color. After five minutes the color is fully developed and the tube may be compared with standards in the comparator block.

All values of this ether extraction method lie within 75 to 95 per cent of those obtained by the Marshall method, using a photocolormeter. Chart 1 shows the departure from the actual values. The average yield is  $81 \pm 8$  per cent, based on 50 determinations. Repeated analyses on the same sample yielded identical checks.

The quantitative estimation of sulfapyridine depends upon the formation of a purplish red azo dye. This diazotization may be completed in approximately one minute and the full color developed in five minutes, as shown in Chart 2.

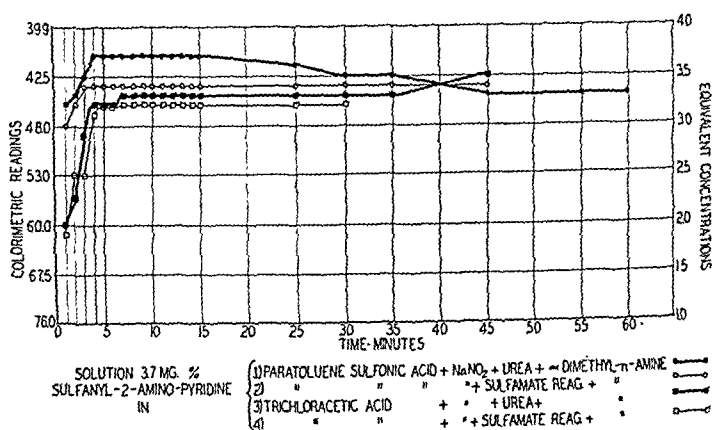


Chart 2.—Rate of color development. When testing for sulfapyridine, readings taken with a photoelectric colorimeter show that complete development of color occurs in five minutes.

The rate of color formation after coupling with  $\alpha$ -dimethylnaphthylamine, the comparative action of ammonium sulfamate and urea on the destruction of the excess nitrite, and the value of trichloroacetic acid and paratoluenesulfonic acid as media for the reaction, were studied. With a photoelectric colorimeter results were obtained which indicated that either group of reagents may be used. Urea and trichloroacetic acid are recommended because they are cheaper and more readily obtainable.

(Note.—While ether does not extract sulfapyridine from watery solutions or urine as efficiently as it does from blood, it extracts sulfapyridine well from cerebrospinal fluid.)

#### PREPARATION OF COLOR STANDARDS

Color standards may be readily prepared using phenol red. They are calibrated so that the value of sulfapyridine obtained from the sample corresponds to the true value. Dissolve 0.0075 Gm. of phenol red in 100 c.c. of distilled water. To a series of 5 tubes, add 3.9 c.c. of fifteenth molar potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) and 6.1 c.c. fifteenth molar secondary, sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ). Then follow with 0.2 c.c. of normal sodium

hydroxide (NaOH) and phenol red as indicated. The volume of the phenol red should increase progressively in the different test tubes.

AMOUNT OF PHENOL RED	AMOUNT OF SULFAPYRIDINE (TRUE VALUE) INDICATED
0.22 c.c.	Concentrations up to 4 mg. per cent
0.30 c.c.	Concentrations up to 7 mg. per cent (usual in blood of recovered pneumococcal pneumonia patients)
0.46 c.c.	Concentrations up to 10 mg. per cent
0.62 c.c.	Concentrations up to 12.5 mg. per cent (desirable in blood of meningitis patients)
0.86 c.c.	Concentrations up to 15 mg. per cent

The phenol red solutions are transferred to test tubes 100 by 12 mm. and sealed. The value of each tube should be marked on or permanently attached to it. The standards should be protected from direct sunlight. New standards should be prepared every six months. In five months our standards had not faded appreciably.

#### SUMMARY

A simplified method for determining sulfapyridine depending upon the ether extraction of sulfapyridine from the blood, is described. The test may be performed in ten minutes and requires simple glassware available in any hospital or office laboratory.

#### REFERENCE

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### A COMPARATIVE STUDY OF THE ERYTHROCYTE SEDIMENTATION TEST WITH THE HELLIGE-VOLLMER (LANGER) MICROSEDI-METER AND THE MODIFIED WESTERGREN METHOD\*

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THE microsedimeter (Hellige-Vollmer)<sup>1</sup> offers a definite advantage in carrying out the erythrocyte sedimentation test in children. The apparatus (Fig. 1) consists of a small box containing 3 graduated pipettes, 3 glass tubes, a celluloid plate, and 2 vials, one with 5 per cent sodium citrate solution and the other with 10 Gm. mercury. The micropipette has no graduation from its tip to mark C, a graduation of 25 mm. from mark C to O, and a mark B, 4 mm. above mark O.

Five per cent sodium citrate solution is drawn up to mark C, and blood from a puncture on an ear lobe or finger tip is drawn up to mark B. The contents of the pipette is then emptied onto the celluloid plate and mixed with the tip of the pipette. The mixture is allowed to flow back by capillary attraction into the pipette, up to mark O. The flat end of the pipette is closed with a finger, the pipette placed into one of the tubes containing a drop of mercury, and then put up in the wooden rack of the apparatus box.

\*From the Pediatric Service of Dr. Bela Schick, Sea View Hospital, Staten Island.

The erythrocyte sedimentation rate is determined by observing the point on the millimeter scale to which the red blood cells fall during one hour.

The method requires only two or three drops of blood, and causes less pain than the macromethod, which necessitates venipuncture. As compared with other micromethods, such as the Landauer<sup>2</sup> and Cutler,<sup>3</sup> it is simpler and less expensive, and is particularly suitable for serial determinations on the same child.

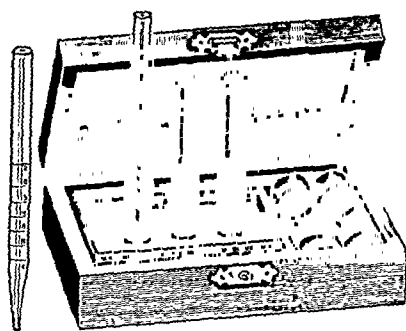


Fig. 1.

In order to examine the reliability of this new method, I compared it with the modified Westergren method used routinely in this institution. I carried out 304 comparative determinations with the micromethod and macromethod. Each point (.), cross (x), or ring (o), in Fig. 2, indicates the sedimentation rate of one blood specimen obtained with the *microsedimeter* (ordinate) as well as with the Westergren *macromethod* (abscissa). An ideal curve was drawn through the spreading points in such a way that these points are situated as near as possible to this ideal curve. With this curve the micrometer values can easily be converted into the corresponding Westergren values.

My observations are in favor of the accuracy of the *microsedimeter* with which method values up to 8 mm. in children, and up to 10 mm. in infants, are regarded as normal. As Fig. 2 demonstrates, these *microsedimeter* values coincide very well with normal Westergren values up to 20 mm.

Another observation is of interest. The time factor in the determination of the blood samples seems to influence the results. In Fig. 2 the sign (.) indicates that both methods were carried out from the same specimen immediately after the blood was taken. The sign (x) indicates that the micromethod was done immediately, but the macromethod only after standing for one to four hours. The sign (o) indicates that both methods were performed simultaneously within one-half to two hours after obtaining the specimen of blood.

The values marked by sign (x) are found to be most distant from the ideal curve. This means that blood standing for several hours gives inaccurate results. Possible reasons for this may be shifting of the blood reaction due to carbon dioxide loss, as well as formation of gross protein aggregates (flocking) which precipitate the red blood cells (J. Loeb).

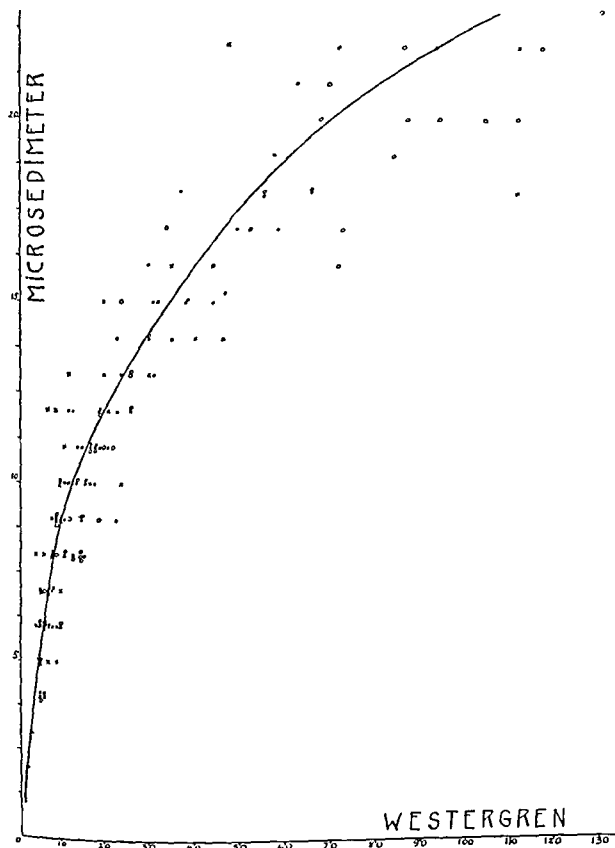


Fig. 2.

## SUMMARY

1. Three hundred four comparative determinations were carried out with the Hellige-Vollmer microsedimeter and the modified Westergren method.
2. The microsedimeter values compare favorably with results obtained with the macromethod.
3. The erythrocyte sedimentation test with the microsedimeter is simple, requires only 2 or 3 drops of blood, and, therefore, can be performed repeatedly on infants and children with the minimum of fear and distress.

4. The standing of blood specimen for several hours gives inaccurate results with the macromethod.

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## COMPARISON OF DARK-FIELD EXAMINATION AND NIGROSINE STAIN IN DEMONSTRATING *TREPONEMA PALLIDUM*\*

NATHAN NAGLE, A.B., AND JOHN GRAUL, ST. LOUIS, MO.

A FAVORABLE report has been made by Dienst and Sanderson<sup>1</sup> on the use of the nigrosine stain in demonstrating *Treponema pallidum*. They state, "This method is much simpler and apparently as accurate as the dark-field examination." With the view of extending the services of the laboratory in the diagnosis of primary syphilis to the practicing physician, we made a comparison of the efficacy of the nigrosine stain and the dark-field examination methods.

Ninety-nine patients, 84 males and 15 females, presenting themselves at the St. Louis Health Division Venereal Disease Clinic, were examined with the dark field, nigrosine stain, and Kahn test.

The technique used in making the nigrosine stain was that recommended by Dienst and Sanderson. The dark-field specimen was usually taken first, followed by at least two preparations for the nigrosine stain. These preparations were stained either on the same day or on the following day. One worker made the dark-field examination and prepared the nigrosine stained smears. Another worker examined these preparations independently, without knowing the results of the dark-field examination.

TABLE I  
COMPARISON OF DARK-FIELD EXAMINATION WITH THE NIGROSINE STAIN ON A GROUP OF 99 PATIENTS HAVING GENITAL LESIONS

NO. PATIENTS	DARK-FIELD EXAMINATION	NIGROSINE STAIN	KAHN TEST
29	Positive	Positive	23 positive 1 doubtful 2 negative 3 no record
49	Negative	Negative	47 negative 2 no record
2	Positive	Negative	Positive
1	Negative	Positive	Positive
1	Positive	Doubtful	Negative
1	Negative	Doubtful	Negative
16	Negative	Negative	15 positive 1 doubtful

\*From the St. Louis Health Division Laboratory.  
Received for publication, July 13, 1939.

Table I gives a summary of the results obtained. Forty-nine patients gave negative tests by all three methods and, therefore, can be excluded from this discussion. Of the 50 persons with primary syphilis, 32 gave positive dark fields, 30 gave positive nigrosine stains, and 41 gave positive Kahn tests. Three patients had positive dark-field examinations while the nigrosine stain method was negative in two and doubtful in the third. On the other hand, one patient had a negative dark field and a positive nigrosine stain. The results indicate a close correlation between both methods in the diagnosis of early syphilis; however, the dark field is the method of choice. Where the dark field is not available to the practicing physician, the nigrosine method is recommended.

#### REFERENCE

1. Dienst, R. B., and Sanderson, E. S.: Use of Nigrosine to Demonstrate *Treponema pallidum* in Syphilitic Lesions, *Am. J. Pub. Health* 26: 910, 1936.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**TUBERCLE BACILLI**, Pathogenicity for Guinea Pigs of, Suspended in Gastric Mucin, Mills, M. A., and Colwell, C. A. *Am. Rev. Tuberc.* 40: 109, 1939.

Sixty guinea pigs were injected subcutaneously with varying doses of tubercle bacilli, equal numbers receiving bacilli suspended in mucin and in saline, for the purpose of determining whether or not any alteration of pathogenicity would occur with the organisms suspended in mucin. No significant differences could be noted with the exception that, of the guinea pigs receiving the smallest doses, those injected with mucinous suspensions developed larger local lesions which showed a greater tendency to ulcerate and discharge their contents. This effect may be explained upon a purely local and mechanical basis. The injected mucin, because of its viscosity, may act temporarily in maintaining a high concentration of bacilli at the site of injection, promoting the development of a more extensive local lesion. With the gradual disappearance of the mucin, the infection progresses in a manner similar to that seen in guinea pigs injected with saline suspensions.

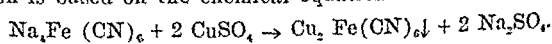
**KIDNEY: Test of Glomerular Function With Sodium Ferrocyanide**, Stieglitz, E. J. *Arch. Int. Med.* 64: 57, 1939.

*Method:* Ampoules of sterile, dry, pure sodium ferrocyanide, containing 0.5 Gm. of the hydrated salt, or about 0.25 Gm. of the anhydrous salt, were employed. Ferrocyanide salts might be decomposed by the gastric acid if taken orally, but on slow intravenous injection no evidences of toxic effects have been observed.

The contents of one ampoule are dissolved in 10 c.c. of sterile distilled water and administered by slow intravenous injection. It is essential that this solution be clear before the injection is started; the salt dissolves rather slowly. Specimens of urine are collected thirty, sixty, one hundred and twenty, and one hundred and eighty minutes after the injection, and their content of ferrocyanide determined by analysis. It is immaterial just how much water is consumed before or during the test; the rate of excretion appears to be independent of the volume of urine. Ample fluid to make possible the prompt voiding of urine at the proper intervals is desirable.

The titration of ferrocyanide is carried out with a 0.4 per cent solution of cupric sulfate (0.004 Gm. per cubic centimeter). Before titration the specimen is acidified with concentrated sulfuric acid. Cupric ferrocyanide is a slightly soluble red salt but is more soluble than the well-known Prussian blue (ferric ferrocyanide). To determine the end point in titration, drops of solution of ferric chloride are placed on a tile, and as the titration proceeds a drop of the unknown is placed in contact with the ferric chloride. If free ferrocyanide is present, Prussian blue is immediately formed; if all the ferrocyanide has already been precipitated as cupric ferrocyanide, a distinct delay occurs before the appearance of this blue color. The end point is, therefore, read when the appearance of the blue is delayed four to five seconds.

The calculation is based on the chemical equation:



The molecular weight of  $\text{Na}_4\text{Fe}(\text{CN})_6$  is 304.08 and that of  $\text{CuSO}_4 \times 2$  is 319.26. If the solution of cupric sulfate contains exactly 0.004 Gm. per cubic centimeter, then 1 c.c. of the solution precipitates exactly 0.0038 Gm., or 1.52 per cent, of the injected dose of 0.25 Gm. of anhydrous sodium ferrocyanide. The exact strength of the cupric sulfate is best first measured by electrolytic methods.

Sodium ferrocyanide, being excreted by the glomeruli, may be safely used as a test substance for determining glomerular efficiency.

The procedure found most applicable is the intravenous injection of 0.5 Gm. of the hydrated salt (representing 0.25 Gm. of the anhydrous salt) freshly dissolved in 10 c.c. of sterile distilled water. Specimens of urine are collected thirty, sixty, one hundred and twenty, and one hundred and eighty minutes after injection, and their ferrocyanide content determined. For ambulatory patients analysis of the first two specimens suffices. Caution must be employed in that the solution *must* be clear before the injection is made.

The excretion rate of ferrocyanide approximately parallels the results obtained with the urea clearance test, the phenolsulfonephthalein test, and the renal concentration test.

Extension of the control series of patients with apparently normal renal function has further clarified what constitutes a normal response. The range of variation is not excessive.

Calculating the rates of excretion on the basis of the ferrocyanide remaining within the body rather than the total dose of salt reveals that the rate of excretion is remarkably uniform throughout the period of study.

In hypertensive arterial disease without other evidence of nephritis, the glomerular function is early and clearly impaired. This depression is most conspicuously manifest during the first hour of excretion. The curve of the percentages of excretion in hypertensive disease is characteristic and suggests that occult glomerular injury depreciates the capacity of the glomeruli to excrete certain solutes promptly. The etiologic relations of these phenomena to hypertensive disease are briefly considered.

The final evaluation of the usefulness and clinical applicability of the test of glomerular function with sodium ferrocyanide can come only after extended investigation, but the present report clearly confirms the impressions gained in the preliminary studies and emphasizes its potential value in clinical study of the relation of glomerular injury to hypertensive disease.

**FOCAL INFECTION and Systemic Disease, Reimann, H. A., and Havens, W. P. J. A. M. A. 114: 1, 1940.**

A review of the case against routine extraction of teeth and tonsillectomy for the purpose of preventing or curing systemic disease shows that the experience of twenty-five years has not justified the practice in the minds of many who have given the matter careful thought. The reasons advanced in favor of the procedure lack controlled clinical and experimental support. By comparing the occasional benefit obtained with the dangers incident to operation, one is led to recommend the procedure only in exceptional cases when evidence of actual local disease is present and its relation to remote or systemic disease is probable.

If abscess or chronic infection around a tooth is present with reasonable certainty, or if the tonsils are actually infected and give rise to repeated attacks of illness, there is no question that surgical measures are necessary in treatment of the local condition. But, in the light of present knowledge, the removal of such local infections in the hope of influencing remote or general symptoms and disease must still be regarded as an experimental procedure not devoid of hazard.

It may be said, therefore, that (a) the theory of focal infection, in the sense of the term used here, has not been proved; (b) the infectious agents involved are unknown; (c) large groups of persons whose tonsils are present are no worse than those whose tonsils are out; (d) patients whose teeth or tonsils are removed often continue to suffer from the original disease for which they were removed; (e) beneficial effects can seldom be ascribed to surgical procedures alone; (f) beneficial effects, which occasionally occur after surgical measures, are often outweighed by harmful effects or no effect at all; and (g) many suggestive foci of infection heal after recovery from systemic disease, or when the general health is improved with hygienic and dietary measures.

**WEIL'S DISEASE, Rapid Presumptive Serological Test for, Brown, H. C. Brit. M. J. Dec. 16, 1939, p. 1183.**

The serologic test for Weil's disease essentially consists in rocking to and fro on a slide for ten minutes small quantities of varying dilutions of the patient's serum in the presence of a heavy suspension of *L. ictero-haemorrhagiae*; this saline suspension is formalized



to a concentration of 0.2 per cent. The preparation of a suitably concentrated antigen for this test is not a simple matter, as *Leptospira* cannot be easily thrown down by means of a centrifuge from the fluid medium in which it is grown. Centrifuging a culture of *Leptospira* in Fletcher's broth at 5,800 r.p.m. for half an hour does not cause any obvious clearing of the supernatant fluid.

The results of the rocking-slide method and the macroscopic have been consistently identical, but in every instance Schüffner's technique has given a titer three times higher than that obtained by either of the other methods. In order to test the sensitivity of the rocking-slide method, two rabbits were immunized with 2 cm. of a broth culture of *Leptospira* administered intravenously. Four days later the sera of these animals were tested by all three methods. The macroscopic and rocking-slide methods gave a titer of 1:10, whereas Schüffner's method gave 1:30. On the seventh day the two former readings were 1:100, and Schüffner's method was 1:300, and on the tenth day 1:1,000 and 1:3,000, respectively.

It is claimed that the rocking-slide method, given a suitable antigen, will produce a result within fifteen minutes of the receipt of the serum, and that it is reliable enough to justify the administration of curative serum.

**THYROID, Histology of, in Exophthalmic Goiter and Hyperthyroidism, Hellwig, A. C.**  
Arch. Path. 28: 870, 1939.

Morphologic studies, including special stains of the colloid and of the finer intracellular structures, fail to reveal fundamental differences between exophthalmic goiter and goiter with hyperthyroidism. Both are variations of a single disease.

Epithelial proliferation and liquefied colloid are the only constant morphologic characteristics of toxic goiter.

Lymphoid tissue alone, without epithelial proliferation, is not a reliable criterion of hyperfunction of the thyroid.

The histologic structure of a diffuse goiter from a patient with the symptoms of exophthalmic goiter is typical in almost all instances. A positive clinical diagnosis can almost always be made from the slide, even after iodine medication.

Hyperthyroidism is caused not only by nodular but also by diffuse colloid goiter. The clinical term "adenomatous goiter with hyperthyroidism" should, therefore, be replaced by "hyperthyroidism."

Microscopic study of nodular goiter does not allow a histophysiologic diagnosis. Toxic and nontoxic nodular goiter are clinical, not anatomic, diagnoses.

The pathologist should use anatomic terms in describing the histologic changes observed in goiter specimens.

The universal adoption of an international classification of goiter is recommended.

**VITAMIN A in the Blood of Normal Adults, Steininger, G., Roberts, L. J., and Brenner, S.**  
J. A. M. A. 113: 2381, 1939.

Semiweekly determinations were made of the amount of vitamin A and carotene in the blood of four experimental and one control subject during a four months' depletion study. The conclusions from these data were supported by those from single determinations of these constituents in the blood of 34 adults, one-half of whom had received a supplement of vitamin A. Biophotometer readings were made of these subjects during the depletion diet and of 31 of the other subjects.

The results of the study show that the amount of vitamin A in the fasting blood is dependent upon the amount in the diet. While the evidence as to whether or not determinations of the vitamin A in the blood are of value in judging nutritional status is contradictory, the data show that consistently low fasting blood values indicate that probably the body stores of vitamin A are being depleted, and very high values indicate that the intake of vitamin A is adequate. The results further show that there is no correlation between the amount of vitamin A in the blood and the biophotometer readings.

**HEMORRHAGE, Chronic, Reaction of Peripheral Blood and Bone Marrow in, and in Essential Thrombopenic Purpura, Limarzi, L. R., and Schleicher, E. M. J. A. M. A. 114: 12, 1940.**

Acute hemorrhage causes a uniform stimulation of the bone marrow with a myeloid, erythroid, and megakaryocytic hyperplasia. The megakaryocytes are of the adult type. The hyperplastic marrow promptly returns to normal.

Chronic hemorrhage causes a similar hyperplasia. The megakaryocytes are usually of the adult type, but there may be many intermediate ones if the hemorrhage is repeated and exhausting. With abolition of the hemorrhages, the bone marrow returns to normal. Platelets are present in large numbers in the bone marrow and in the peripheral blood.

In essential thrombopenic purpura with hemorrhage there is a similar uniform hyperplasia of the bone marrow. In the more chronic cases without significant hemorrhage the myeloid and erythroid hyperplasia may be absent, but the megakaryocytic hyperplasia persists.

In essential thrombopenic purpura the megakaryocytes are of the young form. In the acute phase the promegakaryocytes predominate; in the less acute phases the adult type appears. Hyaline megakaryocytes, which produce the large pseudo-platelets, have been found only in essential thrombopenic purpura. Platelets are reduced equally in the bone marrow and in the peripheral blood.

Splenectomy causes the bone marrow to revert to normal. Unless this reversion is established, the bleeding episodes continue.

The view that essential thrombopenic purpura is a disease due to faulty maturation of the megakaryocytes is favored. Splenectomy removes a factor which is inhibitory to maturation.

The differential diagnosis of the symptomatic purpuras from other hemorrhagic states is not difficult.

The diagnosis of purpuric states can be made most satisfactorily from bone marrow studies. Such studies should lead to a more satisfactory classification of the purpuras.

**PNEUMONIA, Treatment of, Comparison of Results With Specific Serum and Sulfapyridine, Dowling, H. F., and Abernethy, T. J. Am. J. M. Sc. 199: 55, 1940.**

Ninety-six persons with pneumococcus pneumonia treated with serum showed a mortality rate of 16.7 per cent, as compared with a rate of 11 per cent for 136 patients treated with sulfapyridine.

Mortality rates were essentially the same for bacteriemic persons in the two groups, but were definitely lower in the sulfapyridine groups for patients over 40 years and for patients with two or more lobes involved.

While both serum and sulfapyridine are of great value in the treatment of pneumonia, the authors do not believe that conclusions may yet be drawn as to their relative value in its routine treatment.

**TYPHOID VACCINATION, Further Studies of the Intracutaneous Method, Tuft, L. Am. J. M. Sc. 199: 84, 1940.**

Further studies of intracutaneous typhoid vaccination confirmed the author's original observations as to the efficacy of this method, both from an immunologic standpoint as well as in a marked reduction of complete elimination of annoying reactions. In addition, evidence of satisfactory increase of the protective power of the blood and of stimulation of the somatic or O agglutinins was provided.

On the basis of these results, it is believed that the intracutaneous injection of 0.1 c.c., 0.15 c.c., and 0.2 c.c. of the ordinary typhoid vaccine is the most satisfactory of any of the methods for typhoid immunization and should be more widely used. A single dose of 0.1 c.c. seems adequate for immunization of previously vaccinated persons.

**PLATELETS, Enumeration of, in Blood From the Umbilical Cord in Normal Infants,** Hodge, I. G. Bull. Ayer Clin. Lab., Pennsylvania Hosp. 3: 277, 1940.

*Method:* At the time of delivery one clamp was placed on the umbilical cord, which was cut between the clamp and the placenta. Hemorrhage was controlled by manual pressure applied by the operator to the cord. The cut end of the placental segment of the cord was dried with sterile gauze and was held below the level of the perineum in such a way that bleeding was profuse. After a short interval 30 c.c. of blood were collected in a paraffin-lined 50 c.c. beaker. Into a red blood cell hemocytometer, already containing to the 0.05 mark the platelet-diluting fluid recommended by Tocantins, blood was drawn from the beaker until the column of fluid reached the 0.1 mark. The dilution was completed with the same platelet fluid until the mark of 101 was reached. The pipette was shaken gently for ten minutes, and the platelets were counted directly in a counting chamber.

In a small series of 16 infants, satisfactory platelet counts were done on the cord blood according to the method outlined. The average number was found to be 278,000 per c. mm., with extremes of 205,000 and 380,000.

**THE Vi AGGLUTINATION as an Aid in the Detection of Chronic Typhoid Carriers,** Eliot, C. P. Am. J. Hyg. 31: 8, 1940.

The author summarizes the facts about the occurrence of Vi agglutinins in 490 human sera as follows:

The carrier condition is manifested by demonstrable Vi agglutinin in the blood in 95 per cent of proved chronic carriers examined.

The typhoid case frequently shows Vi agglutinin in the blood, probably depending on the stage of the disease, the severity of attack, and other factors to be analyzed in a subsequent report.

The clinically and bacteriologically recovered patient, the inoculated person, and the individual with negative history are alike in that they usually fail to show Vi agglutinins in the blood. Three positive Vi cases were discovered in this group and were proved to be chronic typhoid carriers by cultural methods. Seven other positive Vi cases are so far unexplained.

**MENINGITIS, Spinal Fluid Chlorides in,** Barnes, H. D. South African M. J. 4: 97, 1940.

The chloride content of the cerebrospinal fluid of a number of persons with meningitis has been determined at intervals throughout the course of the disease. Analysis of the results indicates:

The average chloride of the severe cases is reduced to significantly lower values than is that of the mild.

The likelihood of death does not appear to be greater in the cases with lower chloride content of the fluid obtained at first puncture. The degree of association between the extent of reduction of the chloride in the first fluid removed and the subsequent severity of the disease is of doubtful significance.

The chloride curve in pneumococcal meningitis shows no tendency to rise.

When chloride values are within normal limits or, if initially low, the curve rises during subsequent days, the disease is likely to run a mild course, and conversely. The likelihood of death supervening, however, is not significantly greater if the curve falls during the first few days.

**BLOOD: The Choice of an Anticoagulant,** Napier, L. E. Indian J. M. Research 27: 605, 1939.

Packed cell volume estimations were done in duplicate on 50 samples of human blood from various sources. In one side of the centrifuge 2 mg. of dry potassium oxalate were added as an anticoagulant to each cubic centimeter of blood and in the other a mixture of 0.8 mg. of potassium and 1.2 mg. of ammonium oxalate. The results are tabulated.

If Wintrobe's factor,  $\times 1.09$ , to compensate for shrinkage due to potassium oxalate is applied in the case of the former, the means of the two series are almost identical.

It is suggested that this potassium and ammonium-oxalate mixture be adopted as a standard anticoagulant.

**PNEUMONIA, Type Specific Polysaccharide Skin Test in Serum Therapy of, Edwards, J. C., Hoagland, C. L., and Thompson, L. D. J. A. M. A. 113: 1876, 1939.**

In 114 cases of lobar pneumonia caused by type I, II, V, VII, VIII, or XIV pneumococcus, type specific antipneumococcus rabbit serum was used. The reaction of the patient to intracutaneous tests with the type specific polysaccharide from the capsule of a homologous type of pneumococcus was determined before, during, and after the administration of the serum.

One hundred and ten patients gave a negative reaction to the test before serum was given, indicating a low content of circulating antibody. The test proved to be a valuable aid in the more accurate estimation and control of the optimum dose of serum necessary for the successful treatment of the patient.

In 35 cases daily skin tests with the polysaccharide were performed until the patient was discharged from the hospital. In 12 of these cases the reaction became negative before the patients were discharged (seven to nine days), and in 23 it remained positive (nine to nineteen days).

In some cases without complications, a positive reaction to the skin test at the time of admission on the sixth or seventh day of the pneumonia enabled us to withhold treatment with the assurance of the presence of sufficient antibodies to cause a crisis with favorable outcome. Not all patients with spontaneous crises, however, have the excess of free antibodies necessary to cause a positive reaction to the test. Some patients with myocardial damage or cardiac decompensation need serum even though they show a positive reaction.

A persistently negative skin reaction appears to have some value from a prognostic standpoint. In most cases in which a positive reaction occurred after the administration of large amounts of serum the disease terminated fatally. In such cases the blood agglutinins were found in high titer. A skin formerly reactive to polysaccharide may lose its reactivity, even in the presence of free circulating antibody, when the patient is moribund. A positive reaction in a patient with bacteremia does not mean that no more serum is needed. Dermal injection of pneumococcus polysaccharides into human beings may induce subsequent reactivity of the dermal cells; this fact is to be borne in mind in studies of skin sensitivity when repeated intradermal injections of these agents are involved.

The polysaccharide skin test is a valuable, though not infallible, means of measuring the serum required for treatment in a given case of pneumococcic pneumonia.

**SULFAPYRIDINE THERAPY, Renal Complication in, Tsao, Y. F., McCracken, M. E., Chen, J., Kuo, P. T., and Dale, C. L. J. A. M. A. 113: 1315, 1939.**

Five persons with hematuria were associated with administration of sulfapyridine in children. One of the patients died of uremia as a result of complete bilateral urinary obstruction, proved at autopsy.

Hematuria may occur within twenty hours but may be delayed as long as six days after the initial dose of sulfapyridine.

The real mechanism of formation of the hematuria is still unknown, but it may be associated with formation of uroliths.

Cystoscopic examination and ureteral manipulation, and even emergency nephrostomy or renal pyelotomy with probing of the ureter for possible urinary obstruction by a competent urologist, should be made when signs of marked urinary obstruction and anuria develop during the administration of sulfapyridine.

Since children are probably more susceptible to renal complication in sulfapyridine medication, more care should be taken during its administration in pediatric cases.

**PERITONITIS, Primary, in Infants and Children, Ladd, W. E., Botsford, T. W., and Curnen, E. C. J. A. M. A. 113: 1455, 1939.**

In the present clinical study of primary peritonitis, the benefits of early operation and adequate specific therapy have been reflected by a significant reduction in the mortality. The details of treatment remain somewhat elastic and, as pointed out elsewhere, not all the patients were treated according to our present concept of the most effective procedure. The virulence of the organism and the resistance of the patient are two factors in primary peritonitis that always play an important role, which is difficult to evaluate in discussing the results of any type of treatment. Several cases of pneumococcic peritonitis successfully treated with sulfapyridine have been reported recently. This may prove a valuable adjunct to the serum therapy of the disease.

The present program of treatment of primary peritonitis, advocated by the authors, follows:

1. Early incision and drainage of the peritoneal cavity with minimal manipulation.
2. Identification as rapidly as possible of the offending organism obtained from the peritoneal cavity.
3. Immediate postoperative institution of sulfanilamide therapy by hypodermoclysis, continued by oral administration when fluids can be taken by mouth.
4. If a pneumococcus is obtained, substitution of sulfapyridine for sulfanilamide therapy and intravenous administration of type specific antipneumococcus serum.
5. (a) Use of high concentration oxygen and gastric siphonage to relieve abdominal distention; (b) maintenance of adequate fluid intake by parenteral and enteral routes; (c) repeated small blood transfusions to combat anemia and hypoproteinemia.

The high mortality rate in primary peritonitis can be strikingly reduced by early operation for recovery of the offending organism and drainage, followed by adequate treatment with sulfanilamide in the streptococcic group and with type specific serum in the pneumococcic group.

**OVARY, Theca Cell Tumors of, Collins, C. G., Varino, G., and Weed, J. C. J. A. M. A. 113: 1634, 1939.**

Theca cell tumors are a definite clinical entity and, though occurring most frequently at the menopause, they may occur at any age.

From reported cases and our 2 cases of theca cell tumors occurring in young women, it would seem that theca cell tumors may produce menorrhagia in younger women but that amenorrhea of varying periods is the rule. In women near the menopause, menorrhagia is the rule.

Unless fat stains and special studies are made of solid ovarian tumors, especially when associated with amenorrhea or menorrhagia, a number of theca cell tumors will continue to be erroneously diagnosed as fibromas.

**PNEUMONIA, Sulfapyridine in the Treatment of, Plummer, N., and Ensworth, H. E. J. A. M. A. 113: 1848, 1939.**

An analysis of 270 cases of pneumococcic pneumonia reveals that treatment with sulfapyridine resulted in a shortened period of pyrexia, a sterilization of the blood stream, and a low mortality rate.

Blood determinations for sulfapyridine show an irregular but prompt absorption and fail to show a correlation between the blood level and the clinical response.

The incidence of serious toxic reactions is low. Nausea and vomiting are frequent.

Whether serum should also be administered, particularly to seriously ill patients, is still under consideration.

Examination and typing of any available bacteriologic specimens—sputum, blood, spinal or pleural fluid—should not be abandoned. It gives valuable information in diagnosis, in prognosis, and in possible further specific therapy.

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### DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE AND SULFAPYRIDINE BETWEEN CORPUSCLES AND PLASMA IN BOTH HUMAN AND RABBIT BLOOD\*

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IN A STUDY of the therapeutic use of sulfanilamide for gonococcic infections in men<sup>1</sup> the observation was made that the concentrations of both the free and the conjugated drug were almost invariably higher in the corpuscles than in the plasma. This behavior appears to be out of harmony with the idea that sulfanilamide is distributed in proportion to the water content of the tissues.<sup>2</sup> I pointed out that this behavior suggests the possibility of a loose union between the drug and something within the corpuscles or in its membrane.

Furthermore, there appeared to be a correlation between the cure for gonococcic infection and the ratios of the amount of the conjugated form of the drug in the corpuscles to the amount in the plasma. The low ratios were associated with positive cures and the high ratios with failures. For these reasons, and because of the limited number of the individual observations made, and because these observations were all made in connection with only one type of infection, the study on distribution has been extended to individuals treated with sulfanilamide for different infections, including a number of individuals treated repeatedly over extended periods. In addition, the study was extended to include the distribution of sulfapyridine in man infected with different organisms, of sulfanilamide and sulfapyridine in normal rabbits, and of these two drugs when added to human blood in vitro.

*Methods.*—Data presented in Tables I and II were obtained from analyses which were made according to the method of Marshall.<sup>3</sup> All other data were

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TABLE I  
DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF PATIENTS DURING  
TREATMENT FOR VARIOUS INFECTIONS

TREATMENT FOR VARIOUS INFECTIONS AND PLASMA IN BLOOD OF PATIENTS DURING

SEX	INFECTION	IN WHOLE BLOOD				IN PLASMA				PLASMA		IN PLASMA				CORPUSCLES		IN CORPUSCLES				RATIO OF AMOUNT IN	
		FREE SULF.*		CONJUGATED SULF.		TOTAL SULF.		FREE SULF.		CONJUGATED SULF.		TOTAL SULF.		VOL-UMES	FREE SULF.	CONJUGATED SULF.	TOTAL SULF.	per cent	per cent	Free Sulf.	Conjugated Sulf.	100 ML. OF CORPUSCLES	
		mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.											
		mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.											
M	$\beta$ hemolytic streptococci	6.72	1.83	8.55	5.71	1.34	7.05	70.2	59.6	51.4	59.0	29.8	40.4	48.6	41.0	1.59	2.22	1.67					
M	$\beta$ hemolytic streptococci	2.46	1.52	3.98	1.67	0.59	2.26	64.6	43.1	25.0	36.7	35.4	56.9	75.0	63.4	2.38	5.45	3.14					
M	$\beta$ hemolytic streptococci	3.23	1.69	4.92	2.42	1.58	4.0	61.5	46.1	57.4	50.0	38.5	53.9	42.6	50.0	2.59	1.64	2.08					
F	$\beta$ hemolytic streptococci	3.39	2.55	5.94	2.82	1.30	4.12	63.4	52.8	32.5	43.9	36.6	47.2	67.5	50.1	1.55	3.62	2.01					
F	$\beta$ hemolytic streptococci	3.79	1.52	5.31	3.31	1.54	4.85	76.1	66.5	58.0	69.5	23.9	33.5	42.0	30.5	1.60	1.74	1.40					
F	$\beta$ hemolytic streptococci	1.94	0.64	2.58	1.67	0.11	1.78	73.3	62.9	12.5	50.4	26.7	37.1	87.5	49.6	1.62	18.9	2.68					
F	B. coli	6.71	1.01	7.72	4.42	0.58	5.0	66.7	43.8	38.6	43.1	33.3	56.2	61.4	56.9	2.56	3.20	2.63					
M	Streptococcus viridans	7.80	2.51	10.31	7.13	1.47	8.6	58.0	53.0	33.8	48.4	42.0	47.0	66.2	51.6	1.23	2.69	1.48					
M	Streptococcus viridans	4.34	1.96	6.3	3.72	1.21	4.93	72.7	62.4	44.9	57.0	27.3	37.6	55.1	43.0	1.61	3.26	2.01					
F	Gonorrheal arthritis	10.05	1.25	11.3	9.20	0.35	9.55	62.4	57.1	17.6	52.7	27.6	42.9	82.4	47.3	1.25	7.82	1.49					
M	Undulant fever	0.89	0.75	1.64	0.53	0.12	0.65	67.8	40.5	10.7	26.8	32.2	59.5	89.3	73.1	1.47	8.37	2.73					
		3.65	0.65	4.3	2.08	0.42	2.50	65.25	37.2	41.5	37.9	34.75	62.8	58.5	62.1	3.17	2.62	3.08					

\*Sulf. = Sulfanilamide.

\*Sulf. = Sulfanilamide.

prepared from analyses according to the modified procedure of the above method by Marshall and Litchfield.<sup>4</sup>

*Experimental.—Distribution of Sulfanilamide in Human Blood In Vivo.*—Table I shows typical examples of the distribution of sulfanilamide between corpuscles and plasma in a series of individuals, giving sex, type of infection, blood and plasma levels, volumes per cent of plasma, and percentage of the drug in the plasma, volumes per cent of the corpuscles and percentage of the drug in the corpuscles, and the ratio of the concentration of the drug in the corpuscles to that in the plasma. It may be seen that variation in the distribution of the drug between corpuscles and plasma cannot be ascribed to either sex or type of infection. By contrasting the per cent of drug in the plasma and corpuscles with the corresponding volumes per cent, one may readily see that in all the cases cited the per cent of the drug in the corpuscles is noticeably higher than the volumes per cent of the corpuscles, and that the per cent of the drug in the plasma is distinctly lower than the volumes per cent of the plasma, which indicates a decidedly unequal distribution of the drug between corpuscles and plasma, and a consistently higher concentration in the corpuscles. The quantitative difference in the distribution is indicated by the ratios of the concentration in the corpuscles to that in the plasma, ranging from 1.23 to 3.17 for free sulfanilamide, from 1.64 to 18.9 for conjugated drug, and from 1.48 to 3.14 for total drug. It should be noted that the ratio for conjugated drug is generally high when the level of the conjugated drug in the plasma is low, especially when the level in the whole blood is relatively high.

TABLE II  
VARIATION IN DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE BETWEEN CORPUSCLES AND PLASMA OF BLOOD OF PATIENTS DURING TREATMENT

<i>Case 1. Male—Beta hemolytic streptococcus infection</i>								
DAYS TREATED		2	3	5	6	8	9	10
Ratio of amount in	Free sulfanilamide	1.98	1.80	1.74	0.86	1.59	1.90	1.92
	Conjugated sulfanilamide	0.50	0.71	1.14	4.25	2.22	1.30	2.02
100 ml. of corpuscles								
100 ml. of plasma	Total sulfanilamide	1.58	1.56	1.61	1.57	1.67	1.79	1.95
<i>Case 2. Female—B. coli infection</i>								
DAYS TREATED		2	4	5	6	9		
Ratio of amount in	Free sulfanilamide	1.66	1.66	2.26	1.60	2.56		
	Conjugated sulfanilamide	6.29	1.29	0.08	3.79	3.20		
100 ml. of corpuscles								
100 ml. of plasma	Total sulfanilamide	2.11	1.56	1.63	1.81	2.63		

In Table II data from two individuals, one male and one female, indicate typical variations in distribution between corpuscles and plasma over a period of a number of days. While the picture from Table I is sustained, the data here show that the concentrations of both free and conjugated drug may at times be lower in the corpuscles than in the plasma, and that the ratios of the concentration in corpuscles to that in the plasma for both the free and the conjugated drug may vary greatly from day to day with only small changes in the ratio for the total drug, and that there is not any indication for any accumulation of either free or conjugated drug in the corpuscles.



*Distribution of Sulfanilamide in the Blood of Normal Rabbits.*—In Table III data are given to show the distribution of sulfanilamide between corpuscles and plasma of the blood of normal rabbits. Three rabbits, Nos. 1, 2, and 4, received 100 c.c. of an aqueous 0.25 per cent solution of the drug by stomach tube twice a day on two successive days, and on the morning of the third day. Specimens of blood were drawn for analysis on the third day three and one-half hours after the administration of the drug. Number 3 represents a composite of blood from three other rabbits. The drug was administered to these rabbits on three consecutive days in the manner indicated above.

TABLE III

DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF NORMAL RABBITS

RAB- BIT	PLAS- MA	IN PLASMA			CORPUS- CLES	IN CORPUSCLES			RATIO OF AMOUNT IN		
Num- ber	Vol- umes	Free Sulf.*	Conju- gated Sulf.	Total Sulf.	Vol- umes	Free Sulf.	Conju- gated Sulf.	Total Sulf.	100 ml. of Corpuscles		
									100 ml. of Plasma		
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	Free Sulf.	Conju- gated Sulf.	Total Sulf.
1	65.8	65.6	66.4	66.1	34.2	34.3	33.6	33.9	1.13	0.98	1.03
2	65.4	65.5	63.2	63.7	34.6	34.5	36.8	35.2	0.99	1.10	1.08
3†	67.1	63.7	65.6	64.5	32.9	36.3	34.4	35.5	1.13	1.04	1.09
4	71.8	---	---	70.1	28.2	---	---	29.9	---	---	1.08

\*Sulf.=Sulfanilamide.

†Number 3 represents composite blood from three rabbits, hourly specimens from each rabbit over an eight-hour period.

Blood specimens were drawn from each of these rabbits at hourly intervals during the entire third day of treatment. Equal amounts of blood from each rabbit were mixed from each drawing, and all eight separate composites were then mixed for a final composite blood for analysis, so that this sample of composite blood should give a general cross section of the distribution of the drug in the blood of three rabbits for a period of eight hours after several days of continuous treatment with the drug. A comparison of volumes per cent of the plasma with per cent of the drug in the plasma and of volumes per cent of the corpuscles with per cent of the drug in the corpuscles indicates that there is not a large preponderance of the drug in the corpuscles. Number 3, which represents a cross section of the blood of three rabbits over an eight-hour period, shows substantially the same distribution as single specimens from individual rabbits. The ratios of concentration in the corpuscles to that in the plasma show the quantitative aspect of the relationship. On an average there appears to be a slight preponderance of total drug in the corpuscles, but this is shown to be a composite of a slight preponderance of the free drug in the corpuscles and of nearly equal distribution of the conjugated drug for rabbit No. 1; of an almost equal distribution of the free drug and a small preponderance of the conjugated drug in the corpuscles for rabbit No. 2; and of a varying degree of preponderance in the corpuscles of both free and conjugated drug for the composite blood of No. 3.

TABLE IV  
DISTRIBUTION OF FREE AND CONJUGATED SULFAPYRIDINE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF MALE PATIENTS

INFECTION	IN WHOLE BLOOD			IN PLASMA			PLASMA			IN PLASMA			CORPUSCLES			IN CORPUSCLES			RATIO OF AMOUNT IN		
	Free S.P.*	Conju- gated S.P.	Total S.P.	Free S.P.	Conju- gated S.P.	Total S.P.	Vol- umes	Free S.P.	Conju- gated S.P.	Total S.P.	Free S.P.	Conju- gated S.P.	Total S.P.	Free S.P.	Conju- gated S.P.	Total S.P.	Free S.P.	Conju- gated S.P.	100 ml. of Corpuscles	100 ml. of Plasma	Total S.P.
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Meningococcus meningitis	5.00	10.00	15.00	5.72	13.22	18.94	66.4	76.0	87.8	83.8	33.6	24.0	12.2	16.2	0.63	0.28	0.63	0.28	0.38		
Meningococcus meningitis	8.05	2.17	10.22	9.24	2.46	11.70	54.5	62.6	61.8	62.4	45.5	37.4	38.2	37.6	0.67	0.74	0.67	0.74	0.72		
Meningococcus meningitis	8.50	0.95	9.45	9.48	1.33	10.81	61.5	68.6	86.3	70.4	38.5	31.4	13.7	20.6	0.73	0.26	0.73	0.26	0.67		
Meningococcus meningitis	2.89	3.17	6.06	3.27	4.45	7.72	61.6	69.2	85.8	77.9	38.9	30.8	14.2	22.1	0.70	0.26	0.70	0.26	0.45		
Meningococcus meningitis	3.40	1.55	4.95	4.50	1.75	6.25	55.0	73.0	61.9	69.5	45.0	27.0	38.1	30.5	0.45	0.75	0.45	0.75	0.51		
Meningococcus meningitis	4.09	0.55	4.64	4.02	0.76	4.78	72.5	71.2	100.0	---	27.5	28.8	0.0	---	1.07	0.0	1.07	0.0	---		
Influenza meningitis	1.71	1.17	2.88	1.75	1.53	3.28	61.8	63.2	81.2	70.5	38.2	36.8	18.8	20.5	0.94	0.38	0.94	0.38	0.68		
Pneumonia	5.76	0.89	6.65	6.15	1.25	7.40	51.3	54.9	71.9	37.1	48.7	43.1	28.1	42.9	0.87	0.41	0.87	0.41	0.79		
empyema	7.32	0.88	8.20	7.68	8.83	1.15	71.1	74.6	93.2	76.6	28.9	25.4	6.8	23.4	0.84	0.18	0.84	0.18	0.75		
Subacute bacterial endocarditis	0.91	1.30	2.21	0.98	1.82	0.84	66.9	72.5	43.0	55.1	33.1	27.5	37.0	44.9	0.75	2.24	0.75	2.24	2.99		
Osteomyelitis																					

\*S.P.=Sulfapyridine.

*Distribution of Sulfapyridine in Human Blood In Vivo.*—Table IV shows the distribution of sulfapyridine between corpuscles and plasma. The data are arranged in the same manner as in Table I for similar data on the distribution of sulfanilamide. In general, the distribution of sulfapyridine appears to be the opposite of what it is for sulfanilamide, relatively higher concentrations in the plasma than in the corpuscles. This may be seen readily from the per cent values for the drug in the plasma as contrasted with the lower volumes per cent of the plasma, and conversely from the per cent values for the drug in the corpuscles as contrasted with the higher volumes per cent of the corpuscles. The ratios of the concentration in the corpuscles to that in the plasma range from 0.45 to 1.07 for free sulfapyridine, from 0.0 to 2.24 for the conjugated drug, and from 0.38 to 2.99 for the total drug. The ratio is generally higher for the free sulfapyridine than for the conjugated drug. Infrequently it is so high for the free drug as to indicate a slightly higher concentration in the corpuscles than in the plasma. Occasionally an unusually high ratio for the distribution of the conjugated drug may be observed, showing that its concentration may be higher at times in the corpuscles than in the plasma. On the other hand, it is more often entirely absent from the corpuscles. No correlation appears to exist between the type of infection and the distribution between corpuscles and plasma.

TABLE V

VARIATION IN DISTRIBUTION OF FREE AND CONJUGATED SULFAPYRIDINE BETWEEN BLOOD CORPUSCLES AND PLASMA OF TWO PATIENTS

<i>Case 1. Male—Subacute bacterial endocarditis—Streptococcus viridans</i>						
DAYS TREATED		2	3	6	7	8
Ratio of amount in 100 ml. of corpuscles	Free sulfapyridine	0.92	0.88	0.72	0.84	0.84
	Conjugated sulfapyridine	0.0	24.9	0.70	---	0.18
	Total sulfapyridine	---	1.24	0.72	---	0.75
<i>Case 2. Pneumonia with empyema—Pneumococcus infection</i>						
DAYS TREATED		1	2	3	4	5
Ratio of amount in 100 ml. of corpuscles	Free sulfapyridine	0.95	0.88	0.93	0.75	0.94
	Conjugated sulfapyridine	---	0.57	0.30	1.07	0.38
	Total sulfapyridine	---	0.88	0.80	0.84	0.68

That the distribution of both free and conjugated sulfapyridine may vary considerably from day to day, and especially for the conjugated drug, may be seen in Table V, which represents data from two individuals over a period of eight and five days, respectively. The fluctuation is apparently decidedly greater for the conjugated drug than it is for the free sulfapyridine. While there is one exception in each of the two cases, the general rule appears to be sustained that both free and conjugated sulfapyridine are more highly concentrated in the plasma than in the corpuscles, and that this is especially pronounced for conjugated sulfapyridine.

*Distribution of Sulfapyridine in the Blood of Normal Rabbits.*—In Table VI data are given to illustrate the distribution of free, conjugated, and total sulfapyridine in the blood of four normal rabbits with reference to the water content of whole blood, plasma, and corpuscles, and also with reference to the



distribution on the basis of the volumes of the corpuscles and plasma. Sulfapyridine suspended in water was administered by stomach tube on the basis of one gram per kilogram of body weight per day. Each rabbit received two doses of the drug on the first day—one in the morning and one late in the afternoon—and one on the next morning. Blood specimens were drawn from each rabbit three and a half to four hours after the drug was administered on the second day of treatment. The water content of whole blood and of plasma was obtained by using weighed 1 ml. portions of blood and plasma, warming overnight in an oven at  $44^{\circ}$  to  $45^{\circ}$  C., and subsequently bringing to constant weight over calcium chloride in a desiccator. Volumes per cent were obtained from the volumes of corpuscles and plasma of blood, which was centrifuged in calibrated centrifuge tubes

The first part of Table VI gives the volumes per cent of corpuscles and plasma and the grams of water per 100 ml. of blood and per 100 ml. of plasma. These figures are necessary for the calculation of data in the lower part of the table, which gives the levels of the drug in blood and plasma, the concentration of the drug in the water of blood, plasma, and corpuscles, the ratio of the amount in corpuscle water to that in plasma water, and the ratio of the amount in a given volume of corpuscles to that in an equal volume of plasma.

For the sake of comparison the calculation for the concentration of the drug in the water of blood, plasma, and corpuscles was made on the assumption that the drug is held in solution in the water fraction and is not held physically or chemically by components other than water. It can readily be seen from these calculated values that neither free nor conjugated sulfapyridine is distributed between corpuscles and plasma in proportion to the water content. In each of the four rabbits there is only slightly more than half as much free sulfapyridine in 100 Gm. of corpuscle water as in an equal quantity of plasma water. For the conjugated drug the ratio of the content in corpuscle water to that in plasma water varies from 0.21 to 0.94. Apart from the consideration of the water in corpuscles and plasma, the ratios of the amount in corpuscles to that in an equal volume of plasma show for each of the four rabbits a very definite preponderance of both free and conjugated sulfapyridine in the plasma. Thus, there is a definite difference in the distribution between corpuscles and plasma, which cannot be ascribed merely to the difference in water content.

*Distribution of Sulfanilamide and Sulfapyridine in Human Blood In Vitro.*

—In Table VII, a comparison is made of free sulfanilamide and sulfapyridine between corpuscles and plasma of human blood after addition to the blood "in vitro," giving data from three different bloods for each of the drugs. The drugs, dissolved in physiologic saline solution, were added to fresh specimens of human blood. These bloods were then mixed thoroughly, allowed to stand overnight in a cool place, and mixed thoroughly again. Analyses were then made in the usual manner on whole blood and plasma.

It may be seen from the comparison of the per cent of drug in corpuscles and plasma with their volumes per cent that the free sulfanilamide content

tends to be higher in the corpuscles than in the plasma, and that precisely the converse is the case for free sulfapyridine, results which further confirm the preceding data in man.

TABLE VII

SULFANILAMIDE AND SULFAPYRIDINE DISTRIBUTION BETWEEN CORPUSCLES AND PLASMA AFTER ADDITION TO HUMAN BLOOD IN VITRO

SPECIMEN OF BLOOD	PER CENT IN		VOLUMES PER CENT OF	
	CORPUSCLES	PLASMA	CORPUSCLES	PLASMA
<i>Sulfanilamide</i>				
1	34.1	65.9	23.8	76.2
2	23.6	76.5	23.2	76.8
3	34.7	65.3	24.4	75.6
<i>Sulfapyridine</i>				
4	24.8	75.2	27.2	72.8
5	26.1	73.9	30.9	69.1
6	27.5	72.5	34.7	65.3

## DISCUSSION

Results based on clinical material, on experimental data from rabbits, and on human blood "in vitro" indicate that sulfanilamide, and especially the conjugated form, tends to be distributed in blood in such a way that its concentration is generally higher in the corpuscles than in the plasma. In the case of sulfapyridine the relationship is precisely the opposite. The concentration of sulfapyridine, and especially of its conjugated form, is usually decidedly higher in the plasma than in the corpuscles.

From the experimental data on dogs<sup>2</sup> the conclusion was reached that free sulfanilamide is dispersed throughout the body into the various tissues in direct proportion to their water content. It has been shown that conjugation does not occur in the dog.<sup>5</sup> The data presented here on human and rabbit blood and in an earlier paper<sup>1</sup> indicate that free sulfanilamide and sulfapyridine, as well as their conjugated forms, are distributed unequally between corpuscles and water and in a manner out of harmony with the idea of distribution in proportion to water content. A similar conclusion was reached recently by Size<sup>6</sup> in a report on sulfanilamide in human blood. The idea I expressed<sup>1</sup> and the one recently expressed by Size<sup>6</sup> that sulfanilamide may be loosely bound physically or chemically by something in the corpuscles may not be an adequate explanation for the difference in concentration in corpuscles and plasma. It certainly is not for sulfapyridine. It may be that the explanation is to be found chiefly in the difference in the rate of diffusion or absorption through the cell membrane of the corpuscles and other cell and tissue membranes.

While not reported in this paper, the clearance rates and ratios for free and conjugated sulfanilamide were determined for many individuals under treatment, and in a number of cases such determinations were made daily during the entire period of treatment. The clearance rate was generally found to be much higher for conjugated sulfanilamide than for the free drug, confirming previous reports.<sup>1, 7, 8</sup> This difference is probably due to a difference in reabsorption by the tubules, since "in vivo" synthesis of the conjugated

form of the drug does not occur in the kidney, but appears to be limited to the liver.<sup>9, 10</sup> The picture for the conjugated form may be complex, since evidence has been given recently that conjugation may occur with glycuronic acid.<sup>11</sup> However, whatever the form of combination, the facts about relative clearance are unaltered. It seems likely, therefore, that concentrations low in the plasma with reference to concentration in the corpuscles or the entire absence from the plasma may be largely explained by a difference in the rate of elimination by the kidney and passage out of the tissues and the corpuscles into the plasma. This view finds further support from the fact that the very low concentration, in, or the entire absence from, the plasma of free and conjugated sulfanilamide is generally found when the total concentration in the blood is also low. The explanation for the distribution of sulfapyridine, which is precisely opposite to that of sulfanilamide, may also lie chiefly in differences in diffusion or absorption through living membranes. Data for the clearance rates of free and conjugated sulfapyridine are not at hand, but it is a well-known fact that sulfapyridine is much less soluble than sulfanilamide and that acetylsulfapyridine is decidedly less soluble than free sulfapyridine. Nevertheless, differences in diffusion rates through living membranes may not be adequate to explain the slow release of all of the drug from corpuscles in the blood<sup>1</sup> and from other tissues,<sup>12</sup> nor does it appear to explain satisfactorily the distribution of free sulfanilamide and sulfapyridine between corpuscles and plasma of human blood "in vitro."

#### CONCLUSIONS

(1) In "in vivo" studies sulfanilamide, and especially conjugated sulfanilamide, are generally found in higher concentration in the corpuscles than in the plasma of human and rabbit blood.

(2) In "in vivo" studies sulfapyridine, and especially conjugated sulfapyridine, are generally found in higher concentrations in plasma than in the corpuscles of human and of rabbit blood.

(3) Addition of sulfanilamide and of sulfapyridine to human blood "in vitro" results in distribution between corpuscles and plasma in the same manner as stated for "in vivo" studies under (1) and (2).

(4) Relative concentrations of sulfanilamide and of sulfapyridine and of their conjugated forms in corpuscles and plasma vary considerably from day to day, and there does not appear to be any tendency to accumulation of any form of either drug in either corpuscles or plasma.

(5) Sulfanilamide and sulfapyridine and their conjugated forms are not distributed between corpuscles and plasma of human and rabbit blood in proportion to the water content of these blood components.

(6) The type of infection or the influence of sex does not seem to have any effect upon the distribution of sulfanilamide or sulfapyridine between corpuscles and plasma of human blood.

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## A NOTE ON THE LEVEL OF GLUCOSE AND OF NONFERMENTABLE REDUCING SUBSTANCES IN THERAPEUTIC INSULIN SHOCK\*

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IN THE literature published prior to 1933 or 1934 it is generally stated that hypoglycemic shock occurs when the blood sugar falls below 45 mg. per 100 c.c. of blood.<sup>1</sup> This figure refers to the blood sugar as determined by one of the methods which are in common use in clinical laboratories and not to be "true glucose" or, more precisely, the fermentable reducing substances in the blood. The fermentable reducing substances, obtained from the difference in the reducing power of the blood filtrates before and after fermentation, run from 10 to 30 mg. lower than the blood sugars as commonly measured.<sup>1, 2</sup> These 10 to 30 mg. represent the reduction due to nonfermentable reducing substances, most of which come from the corpuscles. It has been found that the amount of these substances is independent of the level of the blood sugar from 50 mg. to 550 mg.<sup>1, 3, 4</sup> and our observations show that even when the glucose is pushed down below 10 mg. the nonfermentable reducing substances remain unchanged in amount (Table I). This table also shows that though these substances do not change with changes in the level of glucose, they do change markedly with rela-

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†Throughout this paper the figures will refer to "milligrams per 100 c.c. of blood," but this expression will be abbreviated to "mg."



TABLE I  
 "BLOOD SUGAR" AND "TRUE GLUCOSE" IN SCHIZOPHRENIC PATIENTS WITH THERAPEUTIC INSULIN SHOCK

"BLOOD SUGAR" AND "TRUE GLUCOSE" IN SCHIZOPHRENIC PATIENTS WITH A LIBERATED PANCREAS									
DATE	SUBJECT AND TREATMENT	METHOD A REVISED FOLIN-WU "LAKED" METHOD		METHOD B "UNLAKED" FOLIN METHOD		"True Glucose," i.e., the values obtained by Method A and Method B	CONDITION OF SUBJECT WHEN SAMPLE WAS TAKEN		
		"Blood Sugar," i.e., total reducing substances in mg. per 100 c.c. of blood	"True Glucose," i.e., fermentable reducing substances in mg. per 100 c.c. of blood	"Blood Sugar," i.e., total reducing substances in mg. per 100 c.c. of blood	"True Glucose," i.e., fermentable reducing substances in mg. per 100 c.c. of blood				
<i>Males</i>									
Jan. 14	Bl. Before insulin	83	59	67	60	60	Fasting and resting		
	Bl. 3 hr. after	59	35	39	32	34	Perspiring but not comatose		
Feb. 4	Ma. Before insulin	80	58	63	55	57	Fasting and resting		
	Ma. 3 hr. after	50	24	29	23	24	Perspiring but not comatose		
	Bl. Before insulin	106	90	91	87	89	Fasting and resting		
	Bl. 3½ hr. after	48	32	24	25	29	Restless, not comatose		
May 3	Ma. Before insulin	108	94	88	86	90	Fasting and resting		
	Ma. 3¾ hr. after	38	23	23	18	20	Comatose for 1 hr.		
	Th. Before insulin	101	89	89	87	88	Fasting and resting		
	Th. 3¾ hr. after	33	21	24	21	21	Restless, not comatose		
	H. 2 hr. after	22	12	11	7	10	Light coma		
	H. 3½ hr. after	20	8	6	2	5	Deep coma		
	S. 2 hr. after	23	12	12	7	10	Light coma		
S. 3½ hr. after	21	8	10	6	7	Deep coma			

TABLE II

"TRUE GLUCOSE," I.E., FERMENTABLE REDUCING SUBSTANCES IN SCHIZOPHRENIC PATIENTS WITH THERAPEUTIC INSULIN SHOCK

DATE	SUBJECT AND TREATMENT	METHOD A. CALCULATED "TRUE GLUCOSE" (= BLOOD SUGAR - 14 MG.)	METHOD B. CALCULATED "TRUE GLUCOSE" (= BLOOD SUGAR - 4 MG.)	AVER- AGE "TRUE GLU- COSE"	CONDITION OF SUBJECT WHEN SAMPLE WAS TAKEN
<i>Males</i>					
May 4	H. Before insulin	86	87	87	Fasting and rest- ing
	H. 2 hr. after 55 units	11	14	13	Light coma
	H. 4 hr. after 55 units	7	6	7	Deep coma
	S. 2½ hr. after 55 units	15	15	15	Perspiring but not comatose
	S. 3½ hr. after 55 units	12	8	10	Light coma
May 5	H. 3½ hr. after 55 units (fluoride)	12	13	13	Deep coma
	H. 3½ hr. after 55 units (oxalate)	11	9	10	Deep coma
	3½ hr. after 55 units	13	7	9	Deep coma
<i>Females</i>					
Aug. 31	Pu. 2 hr., 40 min. after 50 units		9		Deep coma
	Ha. 2 hr., 45 min. after 70 units		4		Moderate coma
	Si. 2 hr., 50 min. after 40 units		4		Moderate coma
	Re. 2 hr., 55 min. after 50 units		5		Light coma
	Mo. 3 hr. after 40 units		8		Deep coma
	Be. 4 hr. after 35 units		10		Light coma, re- covering
	Ho. 4 hr., 5 min. after 50 units		10		Light coma

tively slight changes in the methods of determining blood sugar. For example, on January 14, the day of our first experiment, the reagents were old. On finding that the nonfermentable reducing substances in the filtrates were 24 mg., fresh precipitating reagents were prepared with the result that in all subsequent experiments this figure was 13 mg.  $\pm$  3. The fact that not only the methods of precipitation, but also the methods of measuring the reduction, differ in their specificity, makes it difficult to compare the results obtained by different observers. Depending upon the methods used, the "blood sugar" will exceed the fermentable reducing substances by as little as 2 mg., or as much as 35 mg. The Folin-Wu method, used upon laked filtrates precipitated with tungstic acid, measures as "sugar" approximately 15 to 25 mg. of nonfermentable reducing substances. If 20 mg. is subtracted from the figure of 40 to 50 mg., which is given in the earlier literature, one obtains a figure between 20 and 30 mg. as the probable value of the fermentable reducing substances when shock begins. This agrees, in a general way, with later work, but values outside these limits are frequently reported. Recent work on human beings and animals has shown that during coma it may be much lower: zero in animals<sup>5</sup> and 5 mg. in an infant.<sup>6</sup>

The current treatment of schizophrenia by insulin shocks has afforded an opportunity to follow the total and fermentable reducing substances in the blood

of adults as the glucose is pushed down to levels which produce coma. While it is not certain that normal individuals would have become comatose at the same levels of blood sugar as did these patients, there is, on the other hand, little, if any, reason to believe that the level of coma is altered in schizophrenia. The interest of our observations lies not so much in the level at which coma begins as in the very low levels to which the glucose can fall without causing the death of the individual.

#### METHOD

Blood was drawn from the arm vein of a fasting patient before the injection of insulin and once or twice thereafter, usually at two and one-half or three and one-half hours. Oxalate was used as an anticoagulant in most cases; fluoride in a few. The blood was put in the icebox immediately and precipitated as soon as was convenient, usually within an hour of drawing the last sample. Two methods of precipitation were used, the ordinary Folin-Wu,<sup>7</sup> and the "unlaked" Folin.<sup>2</sup> The reducing substances were measured by the revised Folin-Wu method.<sup>8</sup> The filtrates were then brought to about pH 6.5 and fermented for fifteen minutes with washed yeast at 35° C. and the after-fermentation values were determined. Blanks and controls were done, using the same amount of yeast mixed with water for the blanks and with glucose solution for the controls. The controls showed that the fermentation was complete in less than ten minutes.

The results are given in Table I. The "true glucose" is determined by subtracting the yeast blank from the after-fermentation figure, thereby getting the true after-fermentation value (i.e., the nonglucose reducing substances), and subtracting this difference from the "blood sugar" (i.e., total reducing substances) of the unfermented filtrate.

It has been found<sup>2, 4, 9</sup> that the "unlaked" method is fairly specific for the fermentable reducing substances and includes, on the average, only about 3 or 4 mg. of nonfermentable reducing substances. This is confirmed by the data in Table I. Hence one may calculate the "true glucose" with a fair degree of accuracy by subtracting 4 from the blood sugar, as measured by the unlaked method. The same thing can be done with the laked method, though the amount of nonfermentable reducing substances is larger and less certain, averaging in our experiments (except the first) just over 13 mg., as mentioned above. Table II gives the fermentable reducing substances in the later experiments, calculated from the observed blood sugars by subtracting 14 for the laked method and 4 for the unlaked. The latter half of the table gives the values obtained from 7 female patients from whom only one blood was taken.

#### RESULTS

None of those whose fermentable reducing substances were over 20 mg. were comatose, and all but 2 of those under 20 mg. were comatose, but the number of individuals is too small (18 insulin shocks given to 12 patients) to assign a very definite figure to the level at which coma begins. Furthermore, there was no clear relationship between the apparent depth of the coma and the blood

sugar. In general, the samples were taken at the times when the blood sugar was thought to be at its lowest point, but there are no means of knowing whether we succeeded in getting the lowest point. No untoward aftereffects were seen even when the glucose fell below 10 mg. and remained there for an hour or so, but in these subjects slight injuries to the central nervous system might well pass unnoticed, especially if they were diffuse. This is very near the level at which the damage done to the nervous tissue is so serious and widespread as to prove fatal.

#### SUMMARY

The figures in Table I confirm other workers<sup>2, 4, 5</sup> in showing that the glucose may be determined with a probable error of  $\pm 2$  mg. by subtracting 4 mg. from the blood sugar, as determined upon an unlaked filtrate by the revised Folin-Wu method.

In therapeutic insulin shock in schizophrenic patients the glucose (i.e., fermentable reducing substances) may fall below 10 mg. per 100 c.c. of blood and remain there for periods up to an hour without doing harm of an extent sufficient to be obvious in these subjects. The nonfermentable reducing substances do not change in amount as the blood sugar falls.

We wish to thank Drs. M. Hayman and R. Wilmanns of the Springfield State Hospital for help and facilities in obtaining these samples.

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## VITAMIN C STUDIES

### I. THE EFFECT OF VITAMIN P (CITRIN) ON VITAMIN C DEFICIENT GUINEA PIGS\*

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AN EXTRACT of paprika or lemon juice has been reported to be more effective in the clinical treatment of increased vascular permeability than crystalline vitamin C. Therapeutic potency for the treatment of purpuric conditions was observed in the flavanol glucoside "citrin" fraction of lemon juice. Citrin healed the increased capillary permeability in 3 patients with vascular purpura, but had little, if any, effect on 4 patients with thrombopenic purpura. It exerted a moderate effect in 7 patients with infectious disease, one person with myxedema and 2 persons with diabetes.<sup>1</sup>

Experimentally citrin was found to decrease the number of hemorrhages in scorbutic guinea pigs and prolonged the survival period from 28.5 days for the negative control group to 44 days for the animals given 1 mg. of citrin daily.<sup>2</sup> These findings were not confirmed by the investigations of Zilva<sup>3</sup> nor could they be maintained when the work was repeated by one of the original investigators.<sup>4</sup>

Citrin, which was originally considered a mixture of flavanones, has been resolved into its two chief components, hesperidin and eriodictyol glucoside.<sup>5</sup> Eriodictyol glucoside is demethylated hesperidin and is frequently referred to as eriodictin.<sup>6</sup> It is found in higher concentration in lemons than in oranges.

In this experiment we proposed to study the alleged activity of citrin in scorbutic guinea pigs as a preliminary investigation to its use in the clinical purpuras. A more detailed discussion of the chemistry of isolation, color reactions, and solubilities of these citrin preparations will be found in a separate paper to be contributed by the Research Department of the California Fruit Growers Exchange.

#### EXPERIMENTAL

Four groups of experimental animals (20 guinea pigs each) were used to test the activity of citrin preparations 3, 4, 5, and 6 (Table I) during the months of November, January, March, and June. All animals were given a Sherman-Lamar-Campbell vitamin C deficient diet. Each experimental group was subdivided into four subgroups: Group A, 4 animals, was given 4 c.c. of orange juice daily for an adequate supply of vitamin C and no citrin; Group B,

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4 animals, received the vitamin C deficient diet only; Groups C and D, 6 animals each, were administered different citrin preparations. Citrin in amounts equivalent to that isolated from 100 c.c. of lemon juice was administered daily either orally or intraperitoneally. The animals were weighed twice a week. When the physical condition reached the stage where death seemed imminent, they were killed and studied for the extent and severity of hemorrhagic lesions.

TABLE I  
PROPERTIES OF VARIOUS FRACTIONS OF VITAMIN P  
Isolated by C. W. Wilson

PREPARATION NUMBER	COLOR	FORM	MELTING POINT	BORIC ACID REACTION	Fe Cl <sub>3</sub> REACTION	ASH %	Ba	LEAD
1. B-5837 (Hesperidin)	White	Microscopic needles	257.9 (Decomp.)	None	Weak	None	None	None
2. B-5838	Pale yellow	Microscopic needles	254.6 (Decomp.)	Slight	None	0.25	Small	None
3.* B-5852	Bright yellow	Short micro- scopic needles	241-45 (Decomp.)	+	Emerald green	0.40	Small	None
4.* B-5917 W.S.	Reddish orange glass be- fore vacuum drying	—	—	++	Dirty green, brown with excess	2.1	Large	None
5.* B-6226 W.S.	Greenish brown	Chiefly amorphous few crystals	—	++	Dirty green	9.65	—	—
6.* B-6229	Golden yellow	Amorphous hygro- scopic	—	++	Dirty green	10.70	—	—

\*Preparations tested.  
W.S. = Water soluble.

Citrin preparations 3 and 4, administered by mouth or injected intraperitoneally during the months of November, January, or March, did not prolong the life or ameliorate the hemorrhagic symptoms of scurvy. Twenty-five per cent of these animals, other than the positive control groups that were given orange juice, developed ulcerous lesions in the stomach. The stomach lesions were of two types; one was round, with a clear gelatinous edge, and varied in size from a pin point to the size of the head of a common pin; and the other was moth-eaten in appearance, irregular in shape, occasionally bifurcated, with opaque white edges.

Citrin preparations 5 and 6 injected intraperitoneally into guinea pigs during the month of June did not affect the hemorrhagic diathesis of scurvy. Water-soluble preparation 5 ameliorated the physical deterioration of the animals during the last days of the experiment. Preparation 6 showed no tendency to prolong the life of the animals. Fig. 1 gives a graphic illustration of the effect of preparations 5 and 6 upon the body weight of the June animals.

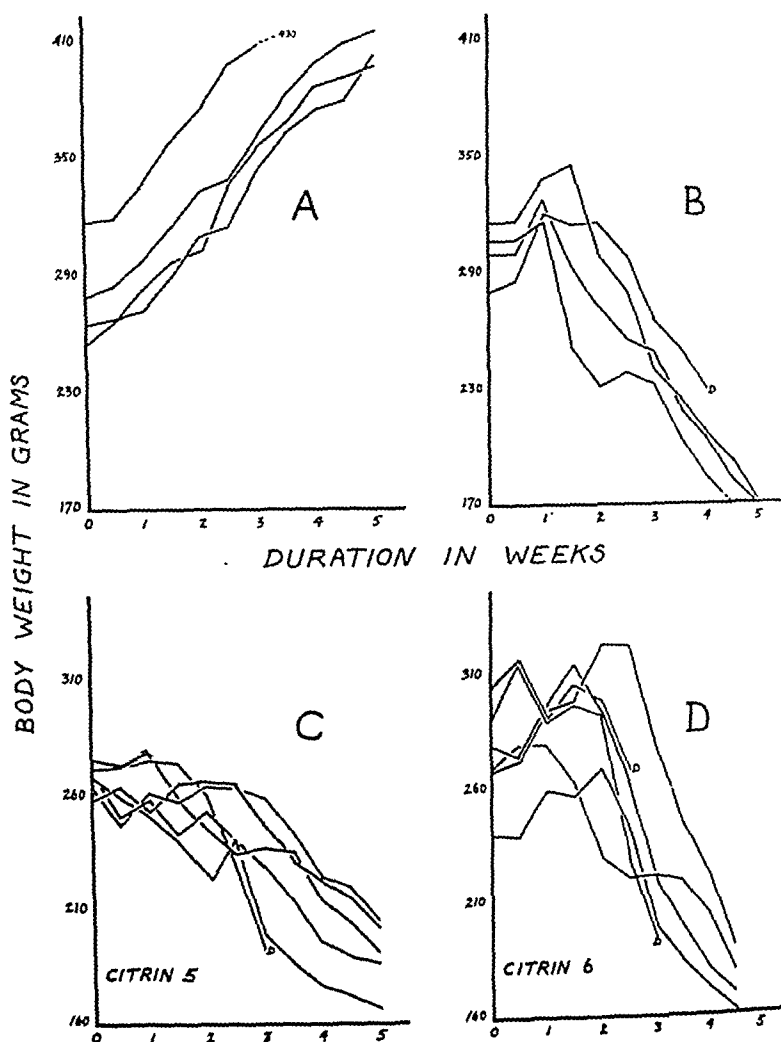


Fig. 1.—Body weight curves for June animals. *D* denotes death of animal.

#### COMMENT

Our observations are in agreement with those of other investigators<sup>3, 4, 6</sup> in that citrin neither decreased the hemorrhagic condition of scurvy nor prolonged the life of the animals. In only one group (Fig. 1, Group C) did citrin improve the physical condition of scorbutic guinea pigs. Since stomach lesions appeared in 20 per cent of the vitamin C deficient animals run during the months of November, January, and March, and not a single stomach lesion appeared in the June experiment, we conclude that winter and summer animals differ in some unknown manner. This unknown factor, probably dietary, protected vitamin C deficient guinea pigs from developing stomach lesions and acted with citrin preparation 5 to improve the general physical condition, but had no effect upon the hemorrhagic diathesis of scurvy.

Clinically, citrin continues to be found effective in capillary wall permeability but not active in thrombopenia. Further investigations are under way in this field.

#### CONCLUSION

Citrin preparations isolated by the technique of Armentano and others,<sup>1</sup> though different in minor respects (details to be published by C. W. Wilson and co-workers), did not decrease the hemorrhagic lesions of the scurvy syndrome or prolong the life of the scorbutic guinea pigs.

We are indebted to C. W. Wilson, of the Research Department of the California Fruit Growers Exchange, for the careful preparation and analysis of the citrin used in this investigation (Table I).

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### URINARY PROLAN EXCRETION DURING A MENSTRUAL CYCLE\*

B. B. BAGBY, JR., M.D., CATAWBA SANATORIUM, VA.

RECENTLY we reported a clinical method for determining the urinary prolan excretion during the menstrual cycle of a normal human female, utilizing various accepted techniques.<sup>1</sup> This paper reports the urinary prolan excretion of a normal woman during one menstrual cycle.

Various investigators have found the pituitary gonadotropic hormone (F. S. H.) present in blood and urine for a few days of the menstrual cycle in comparatively large amounts, whereas it is practically absent the rest of the time. This increased amount usually occurs in the middle of the cycle and is believed to be an indication of ovulation; it does not necessarily occur at a regular time in the cycle. D'Amour<sup>2</sup> and co-workers have shown that the peak may vary from early to late in the period and that in some subjects there may be two separate peaks.

In this experiment both the uterine and the ovarian weights were determined. This was done because Cartland and Nelson<sup>3</sup> have shown that the uterine weight is a more sensitive indicator for small amounts of prolan, although some believe that if dependence is placed in either ovarian or uterine weights, positive results may be missed. Drips and Osterberg<sup>4</sup> use the microscopic appearance of the ovary as an index, but I am not sure that this is necessarily better.

\*From the Department of Clinical Pathology, University of Virginia.  
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I did not attempt to use the two day—6 injections technique of Ascheim and Zondek because I had found, from my previous experiment,<sup>1</sup> that the toxicity of the urinary concentrate due to potassium salts<sup>2</sup> caused rats to die during the course of the experiment. Instead I used the method of Heller,<sup>6</sup> in which urinary concentrate is dissolved in 9 c.c. of water, giving 1 c.c. per dose and 2 doses a day subcutaneously to 21-day-old female rats. The animals were killed on the morning of the sixth day.

The subject was a nurse, aged 19 years, with a normal menstrual history. She had never experienced any intermenstrual pains.

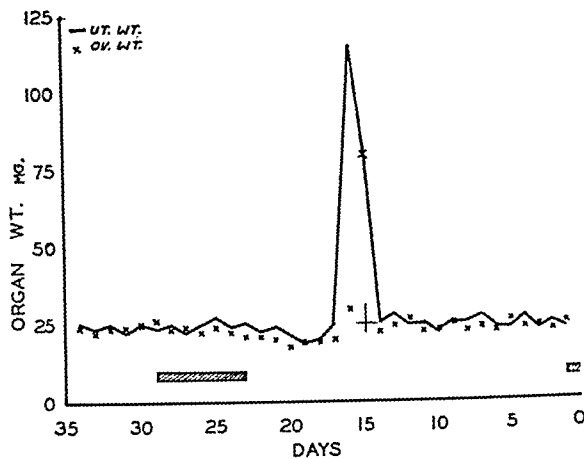


Fig. 1.

In order to make the collecting of urine less tedious I used only the first morning specimen of urine instead of the twenty-four-hour specimen, as suggested previously.<sup>1</sup> This specimen was collected in a bottle containing a small amount of chloroform as a preservative. The urine was then kept on ice until the afternoon or evening when it was acidified to litmus with acetic acid if necessary and then filtered. Four parts of ethyl alcohol were added, the mixture thoroughly mixed and placed in the icebox for at least twenty-four hours. The supernatant fluid was decanted and the precipitate collected by centrifuging. It was then washed twice in ether with careful mixing and dried in air. The precipitate was placed in a small section dish and kept in the icebox until time for injection.

Twenty-four hours before use this precipitate was dissolved in approximately 10 c.c. of distilled water. Ten cubic centimeters were used because the precipitate retained some of the water; I found that this amount of water gave me 9 c.c. of solution to inject. Before injection the remaining precipitate was centrifuged and the prolan solution was decanted. The first injection was given on the afternoon of the first day, and then an injection was made every morning and afternoon until a total of nine injections had been given. On the morning of the sixth day the rats were killed with ether, the uterus was stripped clean, and the fluid, if any, was pressed out on a filter paper moistened with normal saline. The uterus was then immediately weighed in a weighing bottle. Both ovaries were then freed of fascia and weighed together.

It is of some interest to note that none of the rats died during the course of the experiment. The results are shown in Fig. 1. The peak occurred on the fifteenth and sixteenth days prior to the next period (the seventh and eighth days following the cessation of the previous period).

Even though there may be an increase in the excretion of estrogen at the time of the peak, which may cause the increase in the weight of the uterus, the definite increase in the size of the ovaries at this time shows increase in the prolan excretion.

These results again indicate that it is probably unnecessary to take the trouble to weigh the ovaries, since the uterine weight appears to be a far more sensitive indicator. It seems improbable that any estrogenic hormone could be influencing the result, since the uterine weight in this patient and in my previous one<sup>1</sup> increases only when the ovarian weight increases. If estrogenic hormones were coming through the concentration process, we could expect the uterine weight to increase at other times, especially just before menstruation, unaccompanied by an increase in the ovarian weight.

The cost of the experiment, not including the time and overhead, was approximately \$20.00. This is still too expensive a procedure to warrant general clinical application.

#### SUMMARY

The urinary prolan excretion of a normal woman was determined daily for one month, on the first morning specimen, using ovarian and uterine weights of immature rats as indicators. Prolan was found on the fifteenth and sixteenth days prior to the next menses.

I wish to express my indebtedness to Dr. W. E. Bray and to Dr. R. J. Main for their assistance and criticism.

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# THE EFFECT OF SULFANILAMIDE ON THE CROSS MATCHING OF BLOOD\*

## PRELIMINARY REPORT

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WITH THE TECHNICAL ASSISTANCE OF  
FRANCES A. HAMBLIN, B.S., AND LUCY PORCELLI, B.A.

THOUGH many and varied are the side reactions to sulfanilamide, there have been no previous reports in the literature of the effect of the drug on the cross matching of blood. It has been our experience that the blood of patients who had been given sulfanilamide was difficult to cross match.<sup>1</sup> Communication with the medical department of one of the large pharmaceutical houses reveals that they have received many requests concerning such an effect of the drug on the cross matching of blood, but that no study was available.<sup>2</sup>

It was, therefore, decided to determine the effect, if any, of the drug upon the cross matching of blood.

Upon admission to the hospital the blood of each patient was typed and cross matched. A complete blood study—red blood cell and white cell counts, sedimentation time, and blood chloride determination—was also done. Sulfanilamide 15 gr. to 20 gr. with sodium bicarbonate was given every four hours for a period ranging from forty-eight to seventy-two hours. Following this a sulfanilamide concentration test (Marshall's method)<sup>3</sup> was done and the patient was recross matched. Again the sedimentation time and the blood chlorides were determined.

## COMMENT

Although the number of cases reported is too few for any final conclusions to be drawn, certain facts are apparent.

Sulfanilamide per se does not disturb the cross matching of blood.

Accepting the sulfanilamide concentration test as an indication of the ability of the patient to absorb sulfanilamide, it was noted that there was a wide variation in the rate of absorption and excretion of the drug among the patients. One patient who had received 120 grains of sulfanilamide exhibited 15 mg. per 100 c.c. of blood while another patient, under the same dosage, had none in the blood. A patient who had received 300 grains of the drug had a concentration of 3 mg. while one receiving 100 grains had a concentration of 18 mg.

\*From the medical and surgical services of Dr. L. T. Fricke and Dr. G. Kasper, respectively, at the Swedish Hospital.

The Winthrop Chemical Co. supplied the sulfanilamide (Prontylin) used in this study. The blood was furnished by Mixon's Blood Donor Agency.

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TABLE I

NO.	SUB- JECT	SEX	AGE	DISEASE OR CONDITION	BEFORE SULFANILAMIDE				AFTER SULFANILAMIDE					
					BLOOD TYPE	CROSS MATCH- ING	Hb	BLOOD CHLORIDES	SEDIMEN- TATION FAIL IN 60 MIN	TOTAL AMOUNT SULFANIL- AMIDE	SULFANIL- AMIDE CONCEN- TRATION TEST	BLOOD CHLORIDES	SEDIMEN- TATION FAIL IN 60 MIN.	RESULT OF CROSS MATCHING
1	C. L.	♂	54	Fracture left hand	A	Good	98	396	mm.	gr.	mg.	462	mm.	Good
2	C. M.	♀	39	Cardiac neurosis	B	Good	80	396	11	180	16	561	23	Good
3	R. S.	♀	28	Rheumatic polyarthritis	O	Good	84	462	28	240	3	478	21.5	Good
4	N. R.	♂	16	Acute appendicitis	A	Good	94	330	15.5	300	0	360	24	Good
5	J. A.	♀	38	Myositis	A	Good	100	478	3	120	15	396	10	Good
6	P. O.	♀	18	Acute rheumatic fever	O	Good	88	825	22	150	7.6	132	4	Good
7	P. A.	♀	59	Cirrhosis liver	O	Good	80	332	26	195	10	660	26	Good
8	B. S.	♀	28	Rheumatic fever	O	Good	68	330	28.5	165	13	511	1.5	Good
9	W. E.	♀	39	Hypertthyroidism	O	Good	62	363	20	210	17	610	28	Good
										Bad on patients' serum—donors' cells				
10	P. M.	♀	29	Appendicitis	AB	Good	78	330	0	120	7		6	Good
11	B. H.	♂	23	Appendicitis	O	Good	80	396	1	160	8	544	3	Good
12	B. C.	♀	28	Pyelocystitis	B	Good	66	363	8	90	8		12	Good
13	W. R.	♂	49	Urethral stricture	O	Good	84	363	17	100	18	346	20	Good
14	V. E.	♀	49	Chronic polyarthritis	A	Good	73	544	11	120	13	528	25	Good
15	S. C.	♂	52	Hypertrophied prostate	A	Good	82	455	16	160	10	292	30	Good
16	S. L.	♀	23	Upper respiratory infec- tion	O	Good	98	412	3	160	4	380	1	Good
17	L. A.	♀	21	Acute appendicitis	A	Good	78	577	8	120	9	415	6	Good
18	G. B.	♀	33	Influenza pneumonia	O	Good	72	528	4	135	4	396	27	Good
19	M. J.	♂	43	Cirrhosis liver	B	Good	60	511	17	200	5	412	5	Good
20	H. M.	♂	70	Diverticulosis	A	Good	84	346	13	120	0	363	4	Good
21	W. H.	♀	40	Chronic appendicitis and salpingo-oophoritis	B	Good	90	412	23	90	6	396	25	Good
22	D. G.	♂	19	Influenza	B	Good	92	495	2	240	4	495	2	Good
23	W. H.	♀	48	Influenza	B	Bad	82	561	20	180	12	627	10	
										Bad on patients' serum—donors' cells				
24	G. M.	♀	58	Auricular fibrillation	A	Bad on patients' serum— donors' cells	95	464	12	165	8	760	2	Good

In only two cases was difficulty encountered in the cross matching of blood.

In one case (No. 9) the patient's serum agglutinated the cells of the donor. There was no agglutination when the patient's cells and the donor's cells were mixed.

In another case (No. 23) the donor's serum agglutinated the patient's cells. There was no agglutination when the patient's serum and the donor's cells were mixed.

In Case 24 the patient's serum agglutinated the donor's cells before the administration of sulfanilamide. Following the administration of the drug, no agglutination was noted.

It seems, then, that the inability to cross match blood following the administration of sulfanilamide is due to changes in the blood brought about by the disease requiring the use of sulfanilamide rather than to the drug itself.

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5722 FOURTH AVENUE

## RELATION OF LYMPHOCYTES TO THE VIRULENCE OF PNEUMOCOCCI TYPES III AND VII\*

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IT IS rather remarkable that our knowledge of the lymphocyte, especially its function, is so meager, fragmentary, theoretical, and speculative, especially when we consider that the total mass of lymphocytic tissue in the body is greater than that of all the other hemocytic tissue combined, that the lymphocytes are the third most numerous cells in the circulating blood, that lymphocytes are present in almost every inflammatory lesion, toxic malignant growth, and toxic thyroid; and that the lymphocyte is the most universal cell in circulating fluids. The experimental work that has been done bearing on the function of the lymphocyte has led to such conflicting conclusions that at the present time no definite statement can be made which represents a consensus of opinion as to the function or functions of the lymphocyte.

Bergel,<sup>1, 2</sup> from cultural studies, advanced the theory that lymphocytes possess a lipolytic ferment. However, Reed,<sup>3</sup> Caro,<sup>4, 5</sup> and Wiens (as quoted by MacCallum<sup>6</sup>) take exception to the much accepted "Spezifische Fettverdauende, Phagocytierende Tätigkeit der Lymphocyten" as found by Bergel.

\*From the Department of Pathology, University of Oklahoma Medical School.  
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The possible possession of a lipolytic ferment has led to many speculations concerning the role played by the lymphocyte in health and disease. Schaeffer<sup>7</sup> reported that lymphocytes take up fat in the villi and transport it to the lacteals. Many others since Schaeffer have held that the lymphocyte is related to fat metabolism. The statement of Schaeffer seems rather speculative and unproved, and what the relationship, if any, of the lymphocyte to fat metabolism may be is not as yet definitely determined.

Naegeli<sup>8</sup> contends on theoretical grounds that if, as Bergel has stated, the lymphocytes have a special function in secreting a lipoid ferment "a lymphocytosis should be found in all diseases in which the organism exciting the disease contains a lipoid." There would seem to be some confirmation of this theory in the work of Fukushima<sup>9</sup> and Takahashi,<sup>10</sup> who showed that the lipoid isolated from pertussis brought about a lymphocytosis when injected into experimental animals. Opie,<sup>11</sup> however, believes that the lymphocyte contains a protein-digesting ferment which is present only in an acid medium.

Because of the scanty cytoplasm which the cell possesses it has long been thought that lymphocytes are incapable of phagocytosis and for the same reason that they have little power of secretion. Hertzog<sup>12</sup> recently reported that lymphocytes are able to phagocytize. This is as yet unconfirmed and has not been reported under natural conditions, so far as we were able to find.

Murphy and his associates<sup>13-20</sup> undertook extensive investigations into the relation of lymphocytes to resistance in regard to neoplasms and tuberculosis. Their results seem to prove quite definitely that resistance to both transplanted cancer cells and tuberculosis is due to the lymphocytes. It is felt that more critical work should be done in this regard, especially in view of the findings of Loeb<sup>21</sup> in regard to transplant studies. Many other investigations concerning the role of the lymphocyte in relation to tuberculosis and to other diseases, such as whooping cough, seem to bear out the conclusions of Murphy and his associates with respect to other conditions. But none of these authors have given any indication as to the mechanism of this resistance factor.

Because fibroblasts in tissue cultures do better when lymphocytes are added, Carrel<sup>22</sup> believes that the lymphocyte elaborates a growth-promoting substance. Peller<sup>23</sup> also reports that lymphatic tissue is related to growth. But this phenomenon can be better explained on a basis more in harmony with the activities of lymphocytes in other circumstances.

It has been suggested by Bunting and Huston<sup>24, 25</sup> and stated by Maximow,<sup>26</sup> that there is a possibility that lymphocytes bring about a detoxification of bacteria by affixing or absorbing bacterial toxins. Further, Bunting<sup>27</sup> has advanced the hypothesis: "Some mother cell in the lymphoid series produces antitoxins which are then transmitted to her lymphocytic progeny." According to this hypothesis, the lymphocyte would be a passive agent carrying a charge of antitoxic substance. Moor and Newport<sup>28</sup> have experimental evidence that the lymphocyte does have a deleterious effect on pneumococcal toxins. They, as others previously mentioned, feel that this is a phagocytosis phenomenon or an affixing of toxins.

The foregoing citations from the literature prove the chaotic character of our knowledge of the function or functions of the lymphocytes and the need for exact studies of this subject.

Our studies were undertaken (a) to ascertain whether or not lymphocytes do have any effect on the virulence of pneumococci, and (b) if so, to ascertain if possible some indication as to the mechanism of this action.

#### MATERIALS AND METHODS

The chief difficulty in these studies was to secure a sufficient number of lymphocytes. For this purpose the tonsils were selected as a source of lymphocytes because of the ease of securing this material and the great number of lymphocytes in these structures.

Our method for securing lymphocytes from tonsils is similar to that used by Pappenheimer.<sup>29</sup> Tonsils were obtained immediately following removal. The preparation of the suspension took place as quickly as possible in order to avoid any immediate postmortem changes. The tonsils were washed several times in changes of Ringer's solution, and those showing abscesses and other evidences of infection were discarded. Following the washing process, the periphery of the tonsil was seared with the flat side of a heated scalpel, and hot dissecting needles were plunged into the pits. This procedure was carried out for the purpose of destroying as far as possible surface bacteria. The value of this technique was shown by a noticeable decrease in the number of bacteria in the resulting suspension over that of untreated material.

After surface searing the tonsils were again washed in salt solution and finally placed in 10 c.c. of sterile Ringer's solution in an evaporating dish. The tissue was then teased with needles and gently swirled about in the saline during the operation until the resulting solution was a milky white. The suspension was centrifuged to remove tissue fragments. The cells were then washed twice in sterile Ringer's solution.

Part of the suspension of lymphocytes was incubated for twenty-four hours at 37.5° C., then filtered through a sterile Berkefeld candle. This procedure was carried out in order to secure any extracellular metabolite released by the lymphocytes. Thus, two solutions were secured: (1) a suspension of lymphocytes, and (2) a suspension filtrate. Both these solutions were used in the experiments.

An enumeration of the lymphocytes found in the suspensions was done. It was found that they varied in number from 3,500 to 15,000 per c.mm.

Supravital preparations, using Janus green B, neutral red, and brilliant cresyl blue, were examined on a warm stage. The purpose of this procedure was to determine the numbers of living and dead cells. The method and criteria were those used by Pappenheimer.<sup>29</sup> Movement of mitochondria about the nucleus in a semicircular formation was noted. Centrioles were observed dancing about in the cytoplasm and were identified as being quite possibly the bodies described by Gall<sup>30</sup> in the cytoplasm of the living lymphocyte. According to these criteria, lymphocytes may be found alive in these suspensions on the third day of incubation and motile macrophages have been found on the fifth day.

Variations in the size of the lymphocytes were noted, although they appeared to be essentially of the small variety. A few macrophages were observed but they are apparently fewer than expected. The lymphocytes were not observed phagocytizing at any time, even though it has been recently claimed that they may phagocytize under certain conditions (Hertzog<sup>31</sup>). Cells were observed which contained large vacuoles, approaching in some instances the size of the nucleus. Small cytoplasmic bodies, similar to those described by Downey and Weidenreich,<sup>32</sup> were seen budding off from the living cells and were observed in dry preparations.

A gradual formation of a white flocculent precipitate of the suspension was noted. The precipitation began immediately and continued until a clear supernatant liquor resulted. Upon microscopic examination it was seen that these white, flecklike particles were agglomerations of bacteria about lymphocytes which formed the center or nucleus of such groups. The centrioles of the lymphocytes in most cases were very active, hence the cells were alive. The bacteria in a group adhere to each other and thus form larger masses of organisms. It is interesting to note the agglomeration included all forms of bacteria and even spirilla, and that motile bacteria held in the agglomerations were observed attempting to gain a release from the mass. This agglomeration, therefore, was not specific for any type or species of organism.

Whether or not lymphocytes take part in the formation of agglutinins requires further investigation. There is some evidence that lymphocytes may be associated with the production of agglutinins (McMaster and Hudaek,<sup>33</sup> Simonds and Jones<sup>35</sup>). Lowen found a decrease of specific agglutinins during experimental lymphopenia. Williams and Dougherty<sup>36</sup> found an increase in complement following aleuronat injections in the guinea pig. A peripheral lymphocytosis appears to occur at certain stages following the injections of aleuronat. In clinical laboratory procedure most diseases in which serum reactions have the greatest diagnostic value are characterized by a lymphocytosis or lymphadenitis. Therefore, in the observations in regard to agglutinins previously noted, the phenomena may be due to a missing link in the chain, rather than to positive agglutination.

In our studies no evidence of agglutinins was found. The phenomenon described was an agglomeration and required the actual presence of the lymphocyte for the phenomenon to occur.

#### EXPERIMENTS

In order to test the activity of the lymphocyte in a more or less natural state the sputum from pneumonic patients was diluted with sterile normal saline and shaken vigorously to secure a uniform distribution of bacteria. The pneumococci were typed and their virulence ascertained previous to using the sputum in the experiments. The amount inoculated varied from 0.1 c.c. to 0.5 c.c. The total dose of the sputum, regardless of dilution, was made up to 1 c.c., which was the total dosage in all cases. Inoculations were made with a 1 c.c. tuberculin syringe and a short needle.

White mice were used in the experiments because they are extremely susceptible to small doses of virulent pneumococci and because they are so



widely used in the laboratory to test the virulence of the pneumococci in sputum samples.

Control animals were injected with varying amounts of sputum. The experimental animals were injected with sputum which had been added to the lymphocyte suspensions and filtrates. Two sets of experiments were carried out. In one the mixture of sputum and lymphocytes was injected immediately upon being made up. In the other the mixture was allowed to remain in the incubator at 37.5° C. for varying periods of time previous to injection. The reason for this variation was to ascertain whether or not a time relationship at body temperature might affect the results.

The survival time of the animals was taken as a criterion of the detoxification effect of the lymphocytes. The animals were carefully observed constantly during the day and at three-hour intervals during the night. If an animal was found dead at the end of a three-hour period, the former period was taken as the survival time of the animal. Autopsies were made on all animals in an attempt to ascertain as far as possible whether or not death might have been due to a puncture of the intestine. Each animal was marked with dye, and each group of animals was kept separate during the course of the experiment. All pertinent data were kept as to identification, time of inoculation and death, amount injected, and treatment to which the mixture had been subjected.

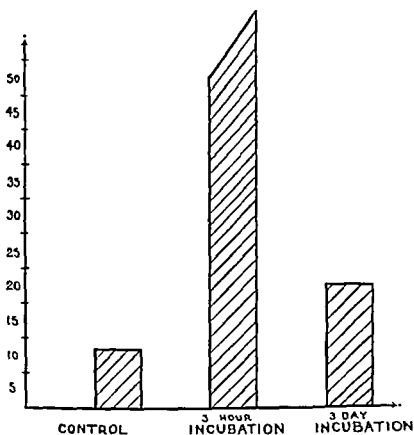
TABLE I  
TYPE VII PNEUMOCOCCUS

ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.5 c.c. suspension	----	----
2	Control	0.5 c.c. suspension	----	----
3	Control	0.5 c.c. sputum	----	7 hours
		0.5 c.c. saline	----	
4	Control	0.5 c.c. sputum	----	7.5 hours
		0.5 c.c. saline	----	
5	Experiment	0.5 c.c. sputum	----	48 hours
		0.5 c.c. suspension	----	
6	Experiment	0.5 c.c. sputum	3 hours	48 hours
		0.5 c.c. suspension		
7	Experiment	0.5 c.c. sputum	3 hours	48 hours
		0.5 c.c. suspension		
8	Experiment	0.5 c.c. sputum	72 hours	18 hours
		0.5 c.c. suspension		
9	Experiment	0.5 c.c. sputum	72 hours	16 hours
		0.5 c.c. suspension		

*Experiment I.*—Type VII pneumococcus was used in the first investigation. The mice, approximately 20 Gm. in weight, were all kept separate during the course of the experiment. The amount of sputum-lymphocyte mixture is noted in Table I. The filtrate was not used in this series. The incubatory period of the sputum and the lymphocyte mixture varied from zero to seventy-two hours. The amount of sputum was kept the same in all injections. Two controls injected with lymphocytes alone showed no untoward symptoms, such as an anaphylactoid response.

There is quite a notable difference in the survival time of the control and the experimental animals (Table I and Graph 1). The three mice which

survived the interval of forty-eight hours were fully as sick as the control mice for the first twenty-four hours, after which time they began to improve, and about the fourth day were quite as normal in appearance and appetite as before the experiment. Mice 8 and 9 which received the sputum and lymphocyte mixture, which had been incubated for seventy-two hours, lived only seventeen hours (about 2.5 times as long as the control). It will be noted that the animals surviving the forty-eight-hour interval lived approximately 6.5 times as long as the control. The average for the group based on the forty-eight-hour interval was thirty-four hours. From the above it seems evident that the lymphocyte had no deleterious effect on the bacteria and that the survival time in these groups depended on the proportion of living lymphocytes present.



Graph 1.—Type VII pneumococcus.

*Experiment II.*—Sputum containing type III pneumococcus was used in the second set of experiments. The procedure followed was essentially that which was used in the experiments with type VII strain, with the exception that the bacteria were given treatment with filtrate as well as with the tonsillar suspension.

The amount of sputum used in the various experiments is noted in Tables II, III, and IV, and the average survival time of the animals in each group is noted on Graph 2.

The sputum was injected in varying amounts: 0.1 c.c., 0.25 c.c., and 0.5 c.c. In each instance filtrate or suspension was added to the sputum and was placed in the incubator for a twenty-four-hour period. The survival time of the animals was carefully noted. Control animals were injected with the same amount of sputum as the test animals, with the exception that physiologic salt solution was added to make the total dose 1 c.c.

TABLE II

ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.5 c.c. sputum	24 hours	7.5 hours
		0.5 c.c. saline		
2	Control	0.5 c.c. sputum	24 hours	8 hours
		0.5 c.c. saline		
3	Experiment	0.5 c.c. sputum	24 hours	12 hours
		0.5 c.c. filtrate		
4	Experiment	0.5 c.c. sputum	24 hours	6 hours
		0.5 c.c. filtrate		
5	Experiment	0.5 c.c. sputum	24 hours	3 hours
		0.5 c.c. filtrate		
6	Experiment	0.5 c.c. sputum	24 hours	3 hours
		0.5 c.c. filtrate		
7	Experiment	0.5 c.c. sputum	24 hours	12 hours
		0.5 c.c. filtrate		
8	Experiment	0.5 c.c. sputum	24 hours	17 hours
		0.5 c.c. suspension		
9	Experiment	0.5 c.c. sputum	24 hours	17 hours
		0.5 c.c. suspension		

TABLE III

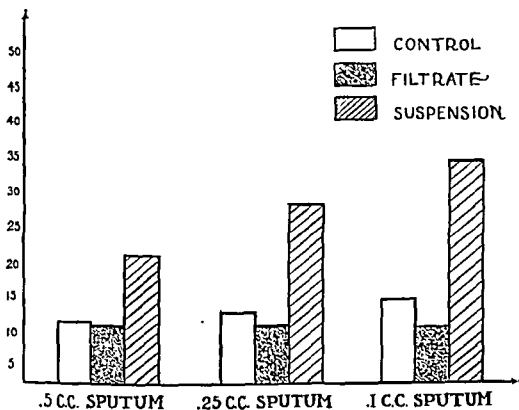
ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.25 c.c. sputum	24 hours	9 hours
		0.25 c.c. saline		
2	Experiment	0.25 c.c. sputum	24 hours	9 hours
		0.75 c.c. filtrate		
3	Experiment	0.25 c.c. sputum	24 hours	6.5 hours
		0.75 c.c. filtrate		
4	Experiment	0.25 c.c. sputum	24 hours	6 hours
		0.75 c.c. suspension		
5	Experiment	0.25 c.c. sputum	24 hours	15 hours
		0.75 c.c. suspension		
6	Experiment	0.25 c.c. sputum	24 hours	21 hours
		0.75 c.c. suspension		
7	Experiment	0.25 c.c. sputum	24 hours	33 hours
		0.75 c.c. suspension		
8	Experiment	0.25 c.c. sputum	24 hours	46 hours
		0.75 c.c. suspension		

TABLE IV

ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.1 c.c. sputum	24 hours	9 hours
		0.9 c.c. saline		
2	Control	0.1 c.c. sputum	24 hours	14 hours
		0.9 c.c. saline		
3	Experiment	0.1 c.c. sputum	24 hours	6.5 hours
		0.9 c.c. filtrate		
4	Experiment	0.1 c.c. sputum	24 hours	8 hours
		0.9 c.c. filtrate		
5	Experiment	0.1 c.c. sputum	24 hours	30 hours
		0.9 c.c. suspension		
6	Experiment	0.1 c.c. sputum	24 hours	36 hours
		0.9 c.c. suspension		
7	Experiment	0.1 c.c. sputum	24 hours	27 hours
		0.1 c.c. suspension		

The individual or average survival time did not deviate materially according to the amount of sputum injected. Hence it may be assumed that the lethal dose was somewhat lower. The averages presented in Graph 2 are based upon the varying amount of sputum injected, although the average survival time in each of the groups is very similar.

The average survival time of the control animals injected with 0.5 c.c. of sputum was 7.5 hours; the filtrate animals averaged 7.3 hours and the suspension animals 17 hours. The control animals receiving 0.25 c.c. of sputum lived 9 hours; the filtrate group 7.3 hours and the suspension group 24.6 hours. The control animals receiving 0.1 c.c. of sputum lived 9 hours; the filtrate group 7.25 hours and the suspension group 26.7 hours. If the survival time of all of the animals in each group are averaged together, it may be observed that the control survival time was 8.5 hours, the filtrate group



Graph 2.—Type III pneumococcus.

7.5 hours, and the suspension group 22.7 hours. Thus, the survival time of the individual groups closely approximates that of the whole. It is also observed that the animals receiving the filtrate of the lymphocyte suspension did not live longer than the control. The small variation on this number of animals is not significant. The animals receiving the suspension lived approximately three times as long as either of the other groups.

This experiment, using both the filtrate and the suspension, was done to ascertain whether the lymphocytes produced some extracellular detoxifying substance as well as to determine whether or not lymphocytes played any part as a defense mechanism in pneumococcus infection.

From the foregoing data it seems to be evident (1) that lymphocytes do not produce any extracellular substance deleterious to pneumococci type III; (2) that living lymphocytes when present do have a detoxifying power on pneumococci type III; and (3) that the theory of an inheritance by the lymphocyte of a detoxifying substance from "some mother cell," as suggested by Bunting, is untenable.

## CONCLUSIONS

A careful analysis of our experiments seems to justify the following conclusions:

1. Lymphocytes play a part in the resistance of white mice to the action of pneumococci types III and VII.
2. There was no evidence in our experiments that lymphocytes produce any extracellular metabolite which plays any part in the phenomena noted.
3. No prolongation of survival time occurred in any of our experiments, except when the living lymphocytes were present.
4. The detoxifying action of lymphocytes on pneumococci in our experiments was in inverse proportion to the virulence of the type.
5. Since the exposure of the pneumococci to lymphocytic suspension filtrate, and since a seventy-two-hour incubation with lymphocytes did not proportionally prolong the survival time of the animals (Graph 1), it would seem that the protective effect of lymphocytes on animals to pneumococci was not due to metabolites or products of autolysis of the cells, as suggested by Moor and Newport,<sup>28</sup> as a possible mechanism.
6. A consideration of these data would seem to indicate that the protective action of lymphocytes to pneumococci was a quantitative one, depending upon the viability of the lymphocytes, and that the mechanism was one of ingestion and intracellular disposition.

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## PROTECTION AGAINST RABIES\*

### I. THE EFFECT OF FREQUENCY OF DOSAGE OF VACCINE UPON IMMUNITY

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A RECENT article by Blatt and co-workers<sup>1</sup> in which twelve deaths from rabies were reported has again impressed upon all the necessity of early adequate immunization as well as sufficient epidemiologic precautions in rabies exposure.

There still exists some controversy as to the best method of acquiring immunity to rabies, especially in reference to the frequency of administration of vaccine. The lack of a satisfactory biological test for rabies immunity has in the main been responsible for the lack of information as to the antigenic response obtained in animals. In recent years certain strains of mice have been found by Webster and Dawson<sup>2</sup> to be susceptible to intracerebral injection of rabies virus. This has offered a method of titrating antiviral serum against rabies virus.

We have been interested in determining the antigenic response to a variable number of daily doses of rabies vaccine. Many clinicians feel that the administration of two injections of rabies vaccine daily gives rise to a more rapid immunity. It has been our impression that the reverse might be accomplished by the too frequent administration of antigen.

#### EXPERIMENTAL

In order to determine the comparative immunity response to varying frequency of doses, 7 groups of rabbits received a variable number of daily injections of rabies vaccine. Each of the 7 groups consisted of 9 rabbits. The first 5 groups I to V received vaccine made by the Harris method; while groups VI and VII received vaccine made by the Semple process. The dosage used was the same as the amount of vaccine administered in human immunization, namely 0.5 c.c. of Harris vaccine and 2.0 c.c. of Semple vaccine. Both vaccines were prepared and used in a fresh state before antigenic potency had an opportunity to deteriorate.

Each of the Harris groups received a total of fourteen subcutaneous injections. Group VI received fourteen inoculations of Semple vaccine, while group VII received a total of twenty-one injections of the Semple vaccine.

Chart 1 gives the schematic plan of injection for each group and also records the deaths from rabies encephalitis. It is interesting to note that in this series the number of deaths from encephalitis is roughly in ratio to the frequency of injections.

\*Eli Lilly Research Fellowship, Indiana University School of Medicine, Indianapolis.  
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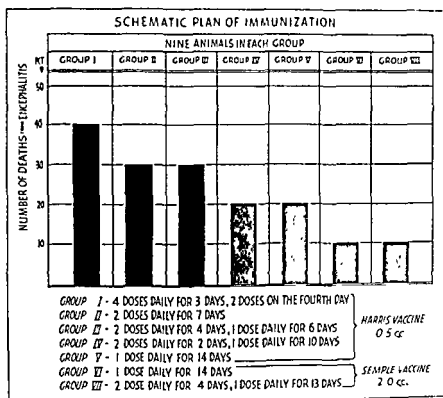


Chart 1.

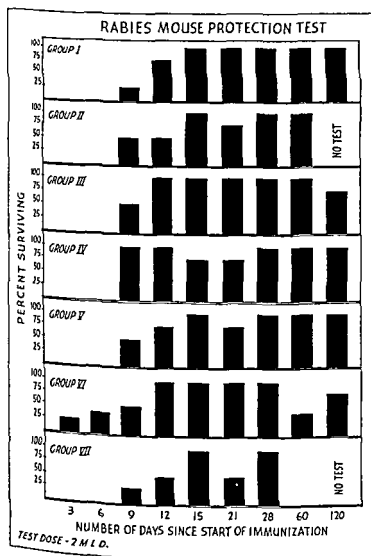


Chart 2.

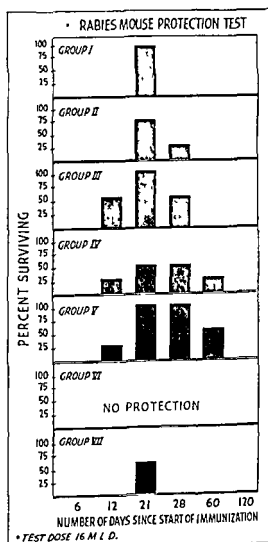


Chart 3.



## IMMUNITY TESTS

Bleedings were made from each group of animals on the third, sixth, ninth, twelfth, fifteenth, twenty-first, twenty-eighth, sixtieth, and one hundred and twentieth days after the first injection of vaccine, with the exception of groups II and VII at 120 days. The sera of the individual groups were pooled to limit variation of animal response.

Each specimen of serum from the respective groups was titrated against two minimal lethal doses of emulsified rabies virus. Two hundredths cubic centimeter (0.02) serum from each bleeding was mixed with 0.02 c.c. of a 1:99 dilution with emulsified virus, which is equal to two minimal lethal doses. The mixture was allowed to incubate at 37° C. in a water bath for one hour. The virus-serum mixture was then injected intracerebrally into four white mice. All series were controlled with varying dilutions of rabies virus mixed with normal rabbit serum. All mice that succumbed did so around the ninth or tenth day. Surviving mice were observed until the twentieth day.

Chart 2 illustrates the protection of the serum from each group when titrated against a test dose of two minimal lethal doses of rabies virus. All controls died by the ninth day. In this chart the percentage of immunity is determined by the percentage of mouse survival. Only group VI (Semple vaccine) demonstrates immunity at the third and sixth days. All groups show some protection at the ninth day.

Groups I and II, which received multiple daily doses of vaccine throughout, did not respond as rapidly as groups III, IV, V, and VI up to the fifteenth day of immunization. It is at that point that sufficient response in all the groups is obtained to protect against two minimal lethal doses almost completely. Group VII has the poorest early response.

Serum-virus neutralization begins to decrease in groups VI and VII as early as the sixtieth day; group VI shows only 33⅓ per cent demonstrable protection, and group VII no protection. In the Harris groups all animals survived at this point. At four months all Harris groups except group III protect 100 per cent, while group VI protects 75 per cent. No tests were made on groups II and VII at four months.

Using only two minimal lethal doses in high dilutions injected intracranially, a small percentage of error may be expected, even though we have a 100 per cent mortality in our controls.

In order to gain a better comparison of the total immunity obtained, the bleedings from all groups were titrated against an infective dose of sixteen minimal lethal doses. The results are shown in Chart 3. In these titrations bleedings on the third, ninth, and fifteenth days were omitted.

Titrating each specimen of serum against sixteen minimal lethal doses we find some protection present as early as the twelfth day in groups III, IV, and V. All of the Harris groups show some protection on the twenty-first day. On the twenty-eighth day all groups except I and VI show some protection. Only groups IV and V show some protection at two months. Protection is not demonstrable in group VI at any time, and there is only 50 per cent protection in group VII on the twenty-eighth day.

It is obvious from Chart 3 that doubling doses with the Harris vaccine decreases the antigenic response, diminishes the rapidity of rise of antibody formation, and shortens the duration of immunity. The attenuated Harris vaccine has given a higher measurable amount of immunity than the killed Semple vaccine, with a longer duration of immunity.

Group V in Table III would seem to demonstrate that best results are obtained from single daily injections of vaccine.

#### DISCUSSION

Many clinicians feel that it is advisable to give multiple daily injections of rabies vaccine in an attempt to stimulate a more rapid response of antibody formation. This is especially true in cases of bites about the head or in close proximity to the central nervous system. The procedure is sometimes used when the starting of treatment has been delayed for various reasons, such as the reluctance of a patient to take treatments or the delayed diagnosis of the biting animal.

It has been our conception that immune response to rabies vaccine would probably resemble that obtained from the administration of certain bacterial antigens. In the production of either tetanus or diphtheria antitoxin small doses of antigen administered at longer time intervals give a higher titer than large doses injected at more frequent intervals. In fact, immune response with those antigens seems to be retarded by large frequent injections. That the same fact is true with rabies vaccine is indicated by the experiments reported here. In Chart 2, with the immune serum titrated against an infective dose of two minimal lethal doses of virus, all groups show a fair percentage of immunity as early as the ninth and twelfth days. Most of the groups are more or less uniform in their response at different intervals of titration.

In order to determine the presence of wide variation in response, tests were run against a much larger infective dose, namely, sixteen minimal lethal doses. Under these conditions real differences are noted, and as shown by Chart 3, there seems to be some ratio of a more pronounced early immune response with a longer duration of immunity, to decreasing the frequency of daily doses.

The immune response in Chart 3 is greater in the groups that received vaccine made by the Harris method than in the groups that received vaccine prepared by the Semple method. This is in accord with Webster's<sup>3</sup> observations in which he states that "vaccines in which virulence has been reduced by phenol are correspondingly less effective as immunizing agents."

There is no information at present concerning the amount of antibody formation needed to protect against rabies. Yet it seems the method of choice in immunization would be that which gives the highest early response with the longest duration of immunity. In our series the groups fulfilling these requirements received but one injection of vaccine daily prepared by the Harris method.

#### CONCLUSIONS

1. Administration of multiple daily doses of rabies vaccine seems to retard immune response.

2. In our experiments an earlier response with a longer duration of immunity was obtained by single daily doses of vaccine made by the Harris method.

I wish to acknowledge my thanks to Mr. W. A. Jamieson for his generous advice and assistance throughout this investigation.

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## TRANSFUSION OF BLOOD FROM ARTIFICIALLY IMMUNIZED DONOR IN THE TREATMENT OF CHRONIC BACILLARY DYSENTERY\*

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THE purpose of this communication is to record the observations with transfusion of blood obtained from artificially immunized donors and used in the treatment of chronic bacillary dysentery. Artificial immunization was produced by the injection of a total of 5 c.c. of the recipient's autogenous vaccine into a suitable donor, without apparent ill effects. An initial dose of 0.1 c.c. of vaccine was given intracutaneously. Three days later, 0.1 c.c. was injected intracutaneously and 0.1 c.c. subcutaneously. Subsequently, the intracutaneous dose remained unchanged, but the subcutaneous one was increased by 0.1 c.c. until 0.5 c.c. was given at one injection.

#### CASE REPORT

Mrs. B. G., 28 years old, was seen in the office on May 6, 1938, because of bloody diarrhea and loss of weight. In February, 1930, immediately after an appendectomy, she passed bright blood in the stool. Two years later she had an acute attack of diarrhea of three weeks' duration. Bloody stools were preceded by generalized abdominal pain. During the spring of the years 1933, 1934, and 1935, she had mild attacks of diarrhea, each episode lasting three months. In 1935 she lost 50 pounds. During the subsequent three years the diarrhea was persistent, and she had 6 to 8 bowel movements daily.

The family history revealed that her brother died of colitis of undetermined origin, and that her mother has had diarrhea for seventeen years. Her blood serum agglutinated Flexner antigens in dilution of 1:320.<sup>1</sup>

The pertinent points in the physical examination were pallor, evidence of loss of weight, and a scaphoid abdomen, with tenderness on moderate pressure in both lower quadrants. The sigmoidoscopic examination revealed a finely granular and easily bleeding mucosa. At the rectosigmoid, the mucous membrane was swollen, thickened, and covered with deep ulcers—a picture of a severe ulcerative colitis.

The bacteriologic studies of the material obtained from these ulcers yielded an atypical Flexner dysentery organism, from which an autogenous vaccine was prepared by Eli Lilly and Company. The stool showed numerous leucocytes and erythrocytes.

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Her blood serum agglutinated Flexner antigens in dilutions of 1:640 on nine different occasions. The blood count revealed hemoglobin 50 per cent (Sahli), 4,770,000 erythrocytes, 10,300 leucocytes, of which segmented polymorphonuclear cells constituted 62 per cent; staff cells 2 per cent; lymphocytes 29 per cent; eosinophiles 5 per cent, and monocytes 2 per cent.

The roentgen-ray examination of the colon, with the aid of a barium enema, showed an ulcerative colitis involving the entire large bowel. There was loss of haustration, narrowing of the lumen, and absence of the mucosal pattern without irritability. The terminal ileum appeared normal.

The patient was not seen again until Sept. 26, 1938. At that time, she had profuse bloody diarrhea, abdominal cramps, and distention, and a fever of 103° F. Two days later she was admitted to the Brooklyn Hospital. Although her general condition improved under supportive treatment and transfusions of blood, the diarrhea continued at a rate of 10 to 15 bowel movements daily. The stools were semiformal or fluid, and mixed with pus, mucus, and blood. Following the administration of autogenous vaccine, the frequency of the diarrhea was reduced to 4 to 6 bowel movements daily.

She was sent home on Nov. 6, 1938. Vaccinotherapy was continued. She showed satisfactory improvement, gained 12 pounds in weight during one month, menstruated for the first time in over six months, and had 4 to 5 bowel movements daily. The stools were semiformal or formed. The temperature ranged from 98.6 to 99° F.

On Jan. 6, 1939, two months after she left the hospital she developed an upper respiratory infection which was associated with a temperature of 102° F. and sharp pains in the wrist, elbow, and shoulder joints. This episode lasted ten days, and was followed by another one on January 21, and again on February 2. During the last episode the diarrhea increased to 8 to 10 bowel movements daily, and the temperature rose to 102.5° F. in the late afternoon and early evening; she experienced abdominal cramps and intermittent pain in the joints. During this time vaccinotherapy on two occasions appeared ineffective, and was, therefore, suspended temporarily.

On Feb. 14, 1939, the patient was readmitted to the Brooklyn Hospital, and an immunotransfusion of 300 c.c. of whole blood was given four days later. On the day of the transfusion she had 4 bowel movements, and continued to have 4 to 5 daily bowel movements with the exception of one day. The temperature remained normal for over a month, but since then has varied from normal to 99.4° F. Her general condition improved satisfactorily, but the pain in the joints was not affected. The blood count four days prior to immunotransfusion was: hemoglobin 68 per cent (Sahli); erythrocytes 3,310,000; leucocytes 20,600, of which polymorphonuclear cells constituted 81 per cent; and the lymphocytes 19 per cent. Three days after the immunotransfusion, the blood count was hemoglobin 76 per cent; erythrocytes 3,940,000; leucocytes 7,250, of which the polymorphonuclears constituted 62 per cent; lymphocytes 24 per cent; and eosinophiles 14 per cent.

To date she has been having 4 to 5 bowel movements daily. The stools are either semiformal or formed. She is gaining in weight, but a nonproductive cough and pains in the left wrist and right shoulder joints still persist.

#### COMMENT

The response of this patient to transfusions of blood was studied in the fall of 1938. At that time the donor had given 500 c.c. of plain blood (September 29) and later 300 c.c. (October 23). The beneficial results from those transfusions could in no wise be compared with those noted after the transfusion of immunized blood from the same donor. The effect on the diarrhea was definite and clear-cut. On the third day after the immunotransfusion, there was a drop, perhaps coincidental, in the number of leucocytes from 20,600 to 7,250. The increase in the hemoglobin and the number of erythrocytes was minimal.

My observations on artificially produced immunotransfusion seem to parallel those noted from transfusions of blood obtained from patients who recovered from bacillary dysentery.<sup>2, 3</sup>

#### SUMMARY

Observations are reported on the effects following a transfusion of 300 c.c. of blood from a donor who was artificially immunized with a vaccine prepared from an atypical Flexner dysentery organism isolated from the ulcers of the recipient's colon.

The practical results obtained in this case and the theoretical basis of this method would appear, in my opinion, to justify an extensive trial of this type of immunotransfusion in order to determine its proper place in therapy.

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## THE EFFECTS OF NICOTINE AND CIGARETTE SMOKE ON PREGNANT FEMALE ALBINO RATS AND THEIR OFFSPRINGS\*

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#### INTRODUCTION

**I**NTEREST in the present study was aroused by one of the questions most commonly asked at the embryologic exhibit of Loyola University at the Chicago World's Fair of 1933-34. The question was: "How does tobacco smoke affect the unborn child?"

Upon review of the literature on the effect of tobacco smoke and nicotine on human beings and experimental animals several limitations were encountered. First, the number of studies done on this problem is relatively small as compared to other problems of equal importance: this is especially true of studies dealing with pregnancy. Second, the findings of various authors are too contradictory to permit the drawing of reliable conclusions. This state of affairs is, at least in part, responsible for the laissez-faire attitude that many physicians take in advising their patients during pregnancy. Third, in many instances the number of animals used in the experiment is too small to gain convincing results. Fourth, far-reaching conclusions have been drawn by some writers from general observation of tobacco users but with no specific study of human beings and animals. This, naturally, has added more confusion to the tobacco problem.

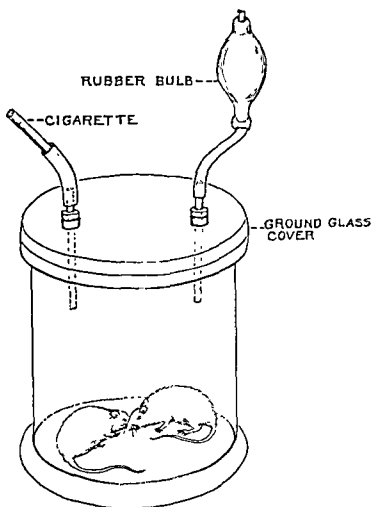
When all of the foregoing facts are considered, the conclusion is that the tobacco problem has not been sufficiently studied. It will require the attention of many investigators before an unbiased answer to the question can be given.

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The question was ripe at the time it was put to us. It is more ripe today and it may prove to be more serious than is generally recognized.

#### MATERIALS AND METHODS

In the selection of our materials and methods we have constantly tried to avoid limitations encountered in the literature. This is especially true of the number of experimental animals. Owing to individual variations, reliable conclusions can be drawn only from the averages of a large number of animals. As a further precaution we have used litter mates of experimental animals for controls.



APPARATUS FOR SMOKING RATS

Fig. 1.

We have used albino rats from the Wistar Institute colony. They have been with us long enough to be perfectly acclimatized. Records of the present colony show that the animals are well fed and are healthy. In all instances young, sexually mature animals were used for experiments. Treatments began from the time of mating and lasted until the weaning of the young.

Careful records were kept of experimental animals and their young. All animals were weighed every second day. Special attention was paid to the length and the course of gestation, to the general health and longevity of the animals, and to the reaction of the mothers toward their young during the time of nursing. The effect of tobacco on the vital functions, and particularly digestion and respiration, was noted. The effect on hair growth was also observed.

The first method was to subject rats to tobacco smoke that would approximate human smoking of about a package of cigarettes a day. For this purpose

an apparatus was devised as shown in Fig. 1. Two holes were drilled in the removable cover of the airtight jar of 6 liters' capacity. One of the holes connected the jar with a smoking cigarette and a bearing-valve rubber bulb was attached to the other. By the operation of the bulb, the chamber of the jar, in which the rats were placed, was filled with as much tobacco smoke as one cigarette could furnish at a time. Only one-third of a cigarette was necessary to fill the jar with smoke. At first three daily exposures were made, but later it was found that the same results were obtained by a daily, three-minute exposure. For the first few seconds of treatment, respiration was noticeably reduced, which, however, returned to about normal so that the animals would make a good many inhalations of the smoke. After a few weeks' treatment the hair became very yellow, due in all probability to the combustion products of the tobacco smoke.

The second method consisted in injection into the rat of solutions of chemically pure nicotine. This method was used to check the efficiency of the first method. Solutions of various strengths were tested, but the final product used was from 1:1,000 to 1:2,000. The injections were made intraperitoneally or subcutaneously, and the amount of the solution used was from 0.5 to 1 c.c. a day per adult rat. Three daily injections were tried at the beginning of the experiment but, as in the case of smoking, this was abandoned in favor of one treatment a day.

The immediate effects on the animals of the two methods are very much the same, except that the injection of nicotine is more severe. Within a minute after injection the animals go into the characteristic nicotine convulsions. The animal stiffens and falls on one side; slight wheezing noises and squeaks are heard, which indicate a type of dyspnea. In a few minutes the violent convulsive condition passes off but the animal still remains on its side or back. If assisted to stand upright, it will fall and roll about, or it may suddenly get up and run aimlessly and fall off the table. The making of rather low sounds or the touching of the animal during the period of recovery will make it jump or go into convulsions again, all of which indicates the reduced threshold of irritability. In some of the treated animals a temporary stage of diarrhea was noted.

In the use of the two methods one may suspect that the mechanics of treatment may be responsible for some of the results. For this reason, some of the control animals were placed in the smoking jar for the usual time and kept there while the air was circulated by the suction bulb. A few of the control animals received injections of neutral distilled water. The results of such treatment were negative in both instances.

#### RESULTS

*Effect on Weight of Young From Treated Mothers.*—In this study the young of both smoked and injected mothers were included. Of the controls there were 113 young, from smoked mothers there were 393 young, and from injected mothers 199 young. The large number in each group was purposely selected to avoid objections, often raised in criticism of former experiments, and to balance out individual variations. The latter, as will be shown later, is of considerable importance in this work.

The results are represented graphically in Fig. 2. The horizontal figures represent the weights in grams; the vertical figures, the ages of the rats in days. The condition of the control animals is shown by the broken line. The circles represent the young from the smoked mothers, and the dots, the offsprings of injected mothers. Each circle or dot represents the average weight of one litter. The same is true of the controls plus the fact that the variations have been removed for clarity.

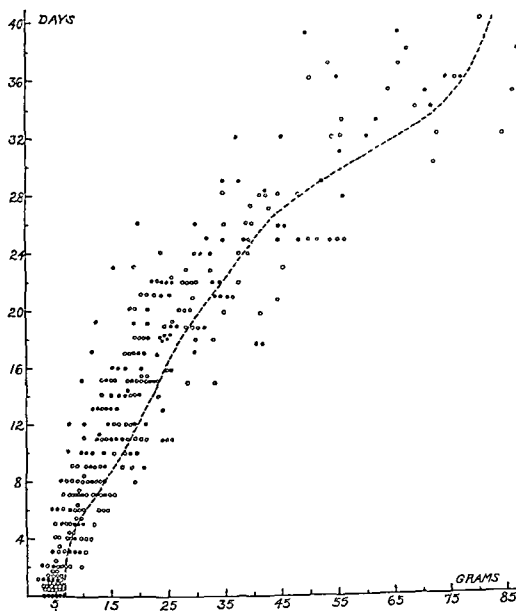


Fig. 2.—Weight record of the young from treated and control mothers. Black dots represent young from injected mothers; circles show young from smoked mothers; broken line denotes young from control mothers. The variations of the litters are omitted for clarity.

It will be seen from Fig. 2 that the young from treated mothers tend to be on the underweight side of the graph. This is true at any given age of the young. Approximately two-thirds of all the young from treated mothers are underweight. The young from injected mothers are slightly more underweight than those from smoked mothers.

*Nicotine Injection of Females During Pregnancy and Lactation.*—A separate series of animals in litter-mate groups were used for this experiment. Each litter was divided as equally as it was feasible into control and experimental animals, of which there were 33 females in the experimental group and 20 in the control group. In each case treatment of the female began at the time of mating and lasted until the end of lactation. The males were not treated. The nicotine



solution used was 1:1,000. Perhaps the best way to report on these experiments is to present the laboratory records of a representative number of litter-mate groups. The records of five such groups, taken at random, are as follows:

First group of 3 litter mates:

1. Female A1, died after forty-three days of injection; progressive increase of weight up to a certain point indicated pregnancy, but there was no delivery.

2. Female A2, delivered 7 young; raised only 3 beyond seven days; all were underweight.

3. Female A3, increase of weight indicated pregnancy, but there was no delivery; this was followed by extreme emaciation.

Second group of 4 litter mates:

1. Female A23, delivered 5 young; 2 died within five days; all were dead within eight days.

2. Females A24 and A25, showed pregnancy weight increase, but there was no delivery.

3. Female A26, delivered 4 offsprings forty-two days after mating; 2 of these were dead by the third day and the last 2 died on the sixth day.

Third group of 4 litter mates:

1. Female A15, delivered an amorphous fetus and some hemorrhagic material; the female died five days after delivery.

2. Female A16, showed the regular increase in pregnancy weight, but died shortly before the time of delivery; on autopsy the uterus was found to be filled with gangrenous fetuses.

3. Female A17, delivered 5 young; 2 of these were stillbirths and the other 3 died on the fourth day.

4. Female A18, delivered on the forty-sixth day; the young were apparently normal.

Fourth group of 4 litter mates:

1. Female A4 died after twenty-six days of injection; there was no appreciable weight increase; early resorption was suspected.

2. Female A5, delivered 7 young; 5 were dead by the fifth day and all by the ninth day.

3. Female A6, delivered 3 young; 2 survived but were underweight.

4. Female A7, delivered 6 young; 4 survived, all were underweight.

Fifth group of 3 litter mates:

1. Female A8, died during the latter part of pregnancy.

2. Female A9, delivered 5 young; all died within six days.

3. Female A10, failed to deliver young.

Of the 30 control females one failed to deliver. All other litter-mate controls delivered normally and raised healthy young.

The results from all injected females may be summarized as follows:

1. The per cent of females losing one or more of their young before weaning was 63.3.
2. The per cent of females losing all of their young before weaning was 33.3.

*Females Exposed to Tobacco Smoke During Pregnancy and Lactation.*—

Owing to the greater variability of results in this series, a larger number of animals seemed necessary. In all, 74 females were exposed to tobacco smoke. Litter mates of these served as controls. As in the former series, treatment was started immediately after mating. For comparison, the laboratory records of three groups of litter mates, taken at random, are presented.

First group of 4 smoked females:

1. Female B20, delivered 9, raised only 6; all were underweight.
2. Female B21, delivered 6, raised 4; all were underweight.
3. Female B22, gave birth to 8 young, of which 4 died and the remaining 4 were underweight.
4. Female B23, delivered 5 and raised 2, apparently normal young.

Second group of 3 smoked females:

1. Female B4, gave birth to 9 young; all were underweight.
2. Female B5, delivered 5; all were underweight.
3. Female B6, delivered 4; all were underweight and dead by the sixth day.

Third group of 3 smoked mothers:

1. Female B65, delivered 7; one died the first day; all were underweight.
2. Female B66, delivered 4; one died at 21 days of age
3. Female B67, delivered 7; all were underweight.

From the foregoing records it is apparent that the smoked mothers and their progeny fare considerably better than the injected animals. The results may be summarized as follows:

1. The per cent of females losing one or more of their young before weaning was 28.3.
2. The per cent of females losing all of their young before the weaning period was 13.5.

*Females Exposed to Tobacco Smoke Prior to Mating.*—This experiment was designed to check the latent effects of tobacco smoke on the mothers. It was desired to treat the females prior to the development of the mammary gland for lactation. If, in so doing, anomalous development of the young can be demonstrated, the injury must reside in the organs other than the mammary gland.

For this purpose 6 females were selected and exposed to daily treatments for three weeks. Upon termination of the treatment, the females were mated and allowed to pass through pregnancy and lactation without further disturbance.

Two litters of young were obtained from each female. The results are summarized as follows:

1. The per cent of females that lost one or more of their young before the time of weaning was 33.3.

2. The per cent of underweight of the young was 25.

*Alterations in the Behavior of Treated Mothers.*—In untreated rat females a certain amount of inborn maternal "responsibility" toward their young can be observed. Toward parturition a nest, although very crude, is usually made by the mother. The young are kept together in the nest and nourished until they can shift for themselves. If some of the young are removed from the group by the attendant, the mothers are often seen to pick them up and carry them back to the nest. In treated mothers these stereotyped patterns of behavior suffer marked alterations. A nest of any kind is seldom built; the young may be scattered all over the floor of the cage, cold and hungry, without the mother paying any attention to them. There is a noticeable indifference on the part of the mothers toward the nursing of their young. The alterations are more common in the injected females than in the smoked ones. They are more severe within several hours after treatment.

Cannibalism is practically unknown in properly fed rat colonies. None has occurred in the control groups during these experiments. In treated animals, however, cannibalism was rather common. It is the second largest factor in the reduction of the litter size, death due to weakness and maternal neglect being the major factor.

Another peculiarity that must be ascribed to the altered maternal behavior is the neglect on the part of the mothers to sever the umbilical cord after birth of the young. In such instances the umbilicus is left to dry in the young until it breaks off of its own accord or is severed by the attendant. In case the attendant performs the neglected surgical operation, the placenta, which is usually devoured by the mother, is left untouched. The number of such instances is relatively small, but none have occurred in the controls.

#### DISCUSSION

The weighing of the females and their young every consecutive day appeared, at first, to be very laborious and time-consuming, yet, as the work progressed, it proved to be one of the best diagnostic means at our disposal. Not only the weight differences between the experimental and control animals was established, but also the course of pregnancy and the developmental process of the young could be followed mathematically.

The majority of the underweight young were underweight at the time of birth. Some, however, were of average weight at the time of birth but lost weight during the period of lactation. Similar observations have been made by Nice,<sup>1</sup> Nakasawa,<sup>2</sup> and Hofstätter.<sup>3</sup> Behrend and Thienes<sup>4</sup> injected nicotine into young rats and observed a decrease in weight but no marked retardation in growth. The decrease in weight was found to be due to the lack of fat deposit. Their findings agree with the weight and growth curves of our treated mothers. In the few instances of extreme emaciation of treated females there is reason to believe that it affected more than the fat deposit. The findings of Behrend and Thienes apply to a certain number of the young that were underweight. The lack of adipose tissue deposit was indicated by their skinniness, angularity of body, and wrinkled skin. The majority of the young of

this group did not only show these characteristics but remained smaller in size than the controls. We are forced to conclude that nicotine poisoning varies with the age of the animal.

In these experiments vaginal smears were not taken, and we have no direct evidence as to the conditions of estrus. However, delayed deliveries have occurred in a number of instances. By this we do not mean a delay of four to six days but delays of two or more weeks in duration. The longest delay of delivery on our records is two months. This seems to indicate that an interference in the reproductive cycle has taken place. Interference with the estrus cycle in rats and mice treated to nicotine has been reported by Lee,<sup>5</sup> Unbehau,<sup>6</sup> and Nakasawa.<sup>2</sup> Since Thienes,<sup>7, 8</sup> Behrend and Thienes,<sup>4</sup> and Staemmler<sup>9</sup> report no effect on the estrus cycle in nicotine treated rats, we assume that estrus disturbances vary with the amount of nicotine administered and the age of the animal in treatment.

Care must be exercised in the appraisal of what we have designated as altered maternal behavior. Apparently this syndrome involves both the mothers and their young. We have found no objective means by which these two can be clearly separated. The disturbances in the digestive, circulatory, and reproductive systems are interpreted as contributory but not major causes of altered behavior of the mothers. Similarly, the interference of nicotine with the development of the mammary glands and lactation, Hatcher and Crosby,<sup>10</sup> Nakasawa,<sup>2</sup> Sodano,<sup>11</sup> and Greiner<sup>12</sup> must be so considered. Since nicotine is primarily a poison of the nervous system (Dixon<sup>13</sup>), and since behavior patterns are chiefly controlled by the nervous system, the major injury must be neural in nature. That this assumption is correct, is indicated by the excitability and restlessness of the mothers and the ease by which they can be thrown into convulsions. That excitability and restlessness afflict most women who have taken to heavy cigarette smoking, is well known to students of the tobacco problem (Campbell,<sup>14</sup> van Hoosen,<sup>15</sup> Greiner,<sup>12</sup> Knopf,<sup>16</sup> and Hofstätter<sup>17</sup>).

Individual resistance to the effects of tobacco smoke offers another point of comparison between treated rat females and smoking human mothers. Some of the litter-mate mothers, receiving the same treatment as the rest, stood the exposure to tobacco products with no apparent detriment. Their young were reared normally, and their weight was normal and sometimes as good as the best of the controls. This was particularly true of the groups that were exposed to tobacco smoke. Likewise there are women who smoke continually and rather heavily and yet they and their offspring remain in what appears to be perfect health. Further investigation will disclose whether these mothers and their offspring are really well or only apparently so.

Nicotine poisoning has been found to occur very often in women employed in the tobacco industry (Klein,<sup>18</sup> Sodano<sup>11</sup>). The case histories of these women are of interest in this connection. Such women are known to suffer from nervousness and insomnia; there are changes in the menstrual cycle and lessened sex desire; they have fewer pregnancies and more abortions and stillbirths; emaciation of the young occurs often, and there is a greater infant death rate.

It will be seen that the parallelism between the histories of women employees of the tobacco industry, and those of our treated rat is strikingly

similar. We interpret that a practical answer to the original question can be found in the case histories of women employed in the tobacco industry and in the histories of women addicted to heavy cigarette smoking.

#### SUMMARY

The following results were obtained in a study of a large number of nicotine-injected and smoked female rats and their offsprings:

1. Two-thirds of all the young of treated mothers were underweight; the young from nicotine-injected mothers were more underweight than those from smoked mothers.

2. The underweight group remained underweight during the entire period of observation; many of the young of this group were undersized and died early.

3. Of the females injected, 63.3 per cent lost one or more young before weaning, and 33.3 per cent lost all of their young.

4. Of the mothers exposed to tobacco smoke, 28 per cent lost one or more of the young before weaning, and 13.5 per cent lost all of their young.

5. Of the mothers smoked prior to mating, 23.3 per cent lost one or more of their young before weaning, and 25 per cent were underweight.

6. In both groups of the treated mothers, temporary sterility, resorption of young in utero, and abortions were noted.

7. Alteration of maternal behavior was observed and consisted of cannibalism and neglect of the young as to care and feeding.

8. A marked parallelism exists between the treated rat females and their young and human mothers and their young in cases where the mother is a heavy smoker or is employed in the tobacco industry.

9. Individual variation is much in evidence.

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## INSULIN SENSITIVITY OF CATS WITH HYPOTHALAMIC LESIONS AND CATS WITH CERVICAL CORD SECTION\*

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THE role of the hypothalamus in the control of carbohydrate metabolism has been investigated by means of insulin sensitivity tests, in cats by Ingram and Barris,<sup>1</sup> and in cats and monkeys by Cleveland and Davis.<sup>2</sup> Following the placing of hypothalamic lesions with the aid of the Horsley-Clarke stereotaxic instrument, these investigators found the animals more sensitive than normal to the hypoglycemic effect of insulin, and less sensitive than normal to the hyperglycemic effect of adrenalin or the diabetogenic effects of anterior lobe extract. Ingram and Winter<sup>1</sup> have recently mentioned similar insulin hypersensitivity in cats with experimental diabetes insipidus.

During the study of temperature regulation and experimental diabetes insipidus in this laboratory, lesions have been placed in the hypothalami of a number of cats. Insulin tests were performed on 76 of these animals, on 21 normal cats, and on 5 cats in which the spinal cord has been sectioned in the lower cervical region. The procedure for each test was as follows:

For the duration of the test the cat was placed on a canvas hammock and secured there in a side-reclining position by means of an arrangement of web straps. This restraint appeared to cause little discomfort, and animals which had been accustomed to such treatment on several days previous to the insulin test showed little excitement and frequently slept much of the time during the experiment. Control tests showed that during such confinement the blood sugar was relatively constant at a level 10 to 20 mg. per cent higher than the average fasting blood sugar of unexcited cats.

After the cat had been on the hammock for at least ten minutes, a blood sample was drawn from the saphenous vein. A sterile solution of insulin was then injected, usually into the saphenous vein of the other leg, although in certain tests the insulin was given subcutaneously over the saphenous vein. Blood samples were drawn thirty, sixty, one hundred and twenty, and one hundred and eighty minutes after insulin injection, and blood sugars (total

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reducing substance) were estimated by the Randles and Grigg<sup>4</sup> modification of the Folin-Wu technique, using 0.5 c.c. samples of blood.

Clotting and fermentation were prevented by the addition of a small amount of a sodium fluoride and thymol mixture (10:1). The dosage of insulin was 0.5 unit per kilogram body weight: the required amount of commercial insulin was diluted with sterile saline solution to a volume of 2.5 to 5.0 c.c., depending upon the size of the cat. Some difference observed in the activity of different brands of insulin led to the selection of Lilly insulin for most of the experiments. The cats were tested after fifteen hours of fasting.

#### NORMAL TESTS

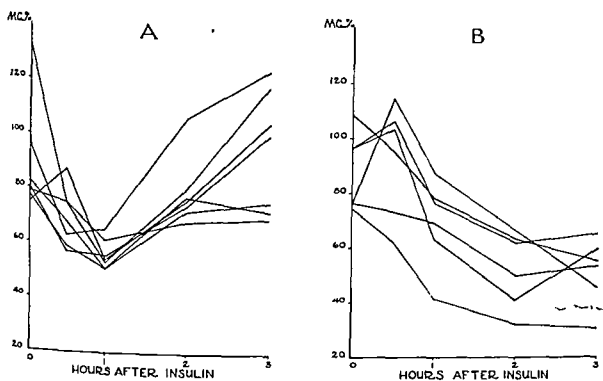
Twenty-two tests were performed on 21 normal cats with 0.5 unit per kilogram of Lilly insulin injected intravenously. In 19 tests the lowest blood sugar level was observed at the end of sixty minutes, while in the other 3 tests the lowest level occurred at the end of thirty minutes. Recovery always took place spontaneously, and in 17 tests the final level was higher than the initial blood sugar. The lowest level noted was 45 mg. per cent; the least amount of final recovery was that of Cat 3 in which the final blood sugar level was 69 mg. per cent, or 87 per cent of the initial level. No external hypoglycemic symptoms were observed in any of the normal animals.

The average initial blood sugar was 94 mg. per cent; the average minimal blood sugar, 57 mg. per cent; the average final level, 109 mg. per cent. At the time of the tests most of these animals had been accustomed to restraint on the hammock.

While this work was in progress a few experiments were carried out with subcutaneous injection of insulin, with results quite different from those obtained when the intravenous method of injection was used. A series of control tests was, therefore, made in which the same animals were tested with insulin injected by both routes, with at least a week's interval between the tests. These experiments showed that hypoglycemia following subcutaneous injection of insulin is more prolonged, that blood sugar reactions are more variable, and that lower levels may be observed than those following intravenous injection of the same amount of the hormone. The results of these tests are presented in Graph 1. From the composite graphs the uniformity between the results of the intravenous experiments is evident, while the blood sugar reactions following subcutaneous injection of insulin were consistent only in that the hypoglycemia was most marked toward the end of the test. Other experiments have suggested that prolonged hypoglycemia may follow the subcutaneous injection of only a part of the dose of insulin when the remainder is then given intravenously.

Three of the 6 cats showed transitory hyperglycemia following subcutaneous administration of the insulin. According to the work of Zucker and Berg,<sup>5</sup> these hyperglycemias may be attributed to an impurity in the insulin; it seems probable that such hyperglycemias also occurred following the intravenous injection of insulin but were undetected in all cases except one, because they occurred in the interval between the first and second samples. Following subcutaneous injection of insulin the hyperglycemic effect was evidently more prolonged.

The cats on which the tests reported in Graph 1 were performed had all been carefully adjusted to handling and confinement on the hammock. The blood sugar averages for the 7 intravenous tests were: initial, 89 mg. per cent; minimal, 55 mg. per cent; final, 94 mg. per cent. The average final level was lower for these 7 tests than for the complete series of 22 tests, suggesting that emotional reactions played a part in the terminal hyperglycemias observed in 17 of the 22 experiments with intravenous insulin. The average amount of hypoglycemia, however, was quite uniform, averaging 55 mg. per cent for the 7 tests of Graph 1, and 57 mg. per cent for the entire series of 22 tests. No external hypoglycemic symptoms were noted following either intravenous or subcutaneous injection.



Graph 1.—Blood sugar responses of normal cats following insulin injection (0.5 u/kg. body weight). A. Intravenous injection; B. Subcutaneous injection.

Brooks<sup>6</sup> commented on the variability of the insulin sensitivity of normal animals following subcutaneously injected insulin. The more uniform results following intravenous injection make that method of administration preferable when the reaction of an animal to experimentally induced hypoglycemia is to be observed.

#### CATS WITH HYPOTHALAMIC LESIONS

Insulin tests were performed on 76 cats in which hypothalamic lesions had been produced for the study of temperature control, experimental diabetes insipidus, or carbohydrate metabolism.\* Operations were performed under nembutal anesthesia, with the aid of the Horsley-Clarke stereotaxic instrument, using either a unipolar or a bipolar needlelike electrode for the production of electrolytic lesions. Histologic verification of the extent of the lesions was made from 50 micra sections stained by the Weil and cresyl violet techniques, or from 10 micra sections stained with cresyl violet.

\*Many of these cats were operated upon by Doctors Magoun, Clark, or Alexander, who kindly allowed their use for insulin experiments.



The results of the insulin tests may be summarized in this way: Thirty-nine animals reacted normally. In 28 animals no hypoglycemic symptoms were observed, but the amount of blood sugar recovery was less than normal; in many cases this could be attributed to postoperative or respiratory infections, or to malnutrition. Hypoglycemic symptoms were observed in 9 cats, associated with inanition, infection, or some other complication in 5 of the 9, but apparently without complication in 4 cats with diabetes insipidus.

TABLE I  
INSULIN SENSITIVITY  
CATS WITH DIABETES INSIPIDUS  
Insulin, 0.5 unit/kg. body weight

CAT NO.	POSTOP. DAY	BLOOD SUGAR (MG. %)					NOTES
		MINUTES AFTER INSULIN					
		0	30	60	120	180	
A 6	83	100	68	58	51	54	Partly subcutaneous
	117	89	69	55	69	99	
A11	100	105	70	65	62		Symptoms of shock
A13	71	122	89	71	37	38	Symptoms of shock
A15	78	86	64	71	70	107	Symptoms of shock Subcutaneous insulin
A16	77	110	85	53	59	44	
A22	106	140	105	77	58	79	
A64	156	127	78	75	69	101	
	162	89	71	60	65	90	
A66	156	88	66	48	48	76	
A70	155	103	83	50	88	115	Symptoms of shock
A88	62	160	117	73		35*	
A90	61	112	38	38	89	12"	

\*150 minute sample.

*Diabetes insipidus:* Tests were performed on 11 cats known to have diabetes insipidus as the result of lesions which interrupted the supraoptico-hypophyseal tracts in the anterior hypothalamus (Fisher, Ingram, and Ranson). The insulin experiments were carried out between postoperative days 61 and 162, when the animals were in good physical condition and weighed more than at the time of operation. The data from the tests are reported in Table I. Six of the 11 cats (A6, A15, A64, A66, A70, and A90) were no more sensitive than normal to the hypoglycemic action of insulin, while in Cat A22 recovery was somewhat delayed, although hypoglycemia was not abnormally marked. The remaining 4 cats were hypersensitive; Cats A11, A13, and A88 showed unmistakable symptoms of insulin shock toward the end of the three-hour test period, although the blood sugar level of Cat A11 at the time the symptoms appeared was evidently not as low as the shock level. Cat A16 was tested with subcutaneous injection of insulin and experienced shock shortly after the end of the test; two weeks later, however, a test performed with intravenous injection of insulin gave normal results.

The hypoglycemic symptoms included muscular flaccidity, relaxation of anal and bladder sphincters, and apathy to ordinary visual, auditory, or cutaneous stimulation. Injection of sterile glucose solution either intravenously or subcutaneously promptly relieved the symptoms in all cases, although more than one injection was sometimes necessary to effect complete recovery.

The lesions of Cats A11, A13, A16, and A88 were located in the medial part of the anterior tuberal portion of the hypothalamus in the region close to the ventral extent of the third ventricle where the supraoptico-hypophyseal tracts lie. This corresponds closely with the description of the lesions reported by Ingram and Barris<sup>1</sup> associated with the disturbance of carbohydrate metabolism: "Bilateral and situated either close to the walls of the ventricle in the region of the filiform nucleus or in the perifornical and subfornical regions of the anterior hypothalamic zone."

*Other tests:* Normal insulin responses were observed after lesions in the anterior portions of the lateral hypothalamic areas, after large transverse lesions at the level of the chiasma, after large midline lesions between chiasma and infundibulum, after discrete bilateral lesions in the fornices, or after discrete lesions in the midline at the level of the median eminence. There was no correlation between disturbance of temperature regulation and insulin responses, and cats in which the paraventricular nuclei were almost completely destroyed reacted as normal.

TABLE II  
BLOOD SUGAR FOLLOWING HYPOTHALAMIC LESIONS

WEEK NO.	TOTAL TESTS	NO. OF CATS	BLOOD SUGARS IN MG %		
			MINIMUM	MAXIMUM	AVERAGE
Preop. control	45	41	65	133	88
1	15	15	83	170	108
2	26	25	65	119	88
3	20	20	69	110	85
4	17	16	67	119	81
5	17	15	67	100	79

From this series of experiments it was not possible to assign to any hypothalamic structure definite function in the control of carbohydrate metabolism. The 4 hypersensitive animals showed similar lesions in the medial portion of the anterior tuberal hypothalamus, but other cats with lesions in the same area reacted normally to insulin; and one animal with destruction of almost the entire anterior hypothalamus reacted normally when tested 19 days after the second stage of a two-stage hypothalamic operation.

To supplement the data obtained from insulin tests, estimations were made of fasting (fifteen hours) blood sugar levels of some of these animals both before operation and for a period of 5 weeks after operation. In Table II the data from these analyses are arranged according to the week in which the tests were performed, with the preoperative control tests listed at the top of the series. From a preoperative average of 88 mg. per cent, the blood sugars of the cats rose to an average of 108 mg. per cent during the first postoperative week and then gradually fell to 79 mg. per cent at the fifth week after operation. The immediate postoperative rise may be attributed to the effect of the operations, for the minimal as well as the maximal blood sugars noted during the first week were higher than the control levels. On the other hand, the ultimate decline of the average levels to a figure 9 mg. per cent lower than the control

average does not represent a tendency toward spontaneous hypoglycemia, for the minimal levels were almost the same before operation as during the second, third, fourth, and fifth postoperative weeks. The lower average was coincident with a similar decline in the maximal levels observed, and probably represented a tendency toward emotional stability in the cats as they became more accustomed to being handled and placed on the hammock. As a result, five weeks after operation there was less emotional hyperglycemia associated with the taking of the samples. These data confirm the observation of postoperative hyperglycemia made by Barris and Ingram<sup>5</sup> following the production of hypothalamic lesions, but during the first five postoperative weeks this series of cats did not show the hypoglycemia which they described.

#### SPINAL CATS

Insulin tests were performed on spinal cats by Brooks,<sup>6</sup> but the animals he used were operated upon only a week previous to the tests and were not eating readily at the time they were utilized for the experiments. His results, however, indicated that such cats were able to recover normally from small doses of insulin.

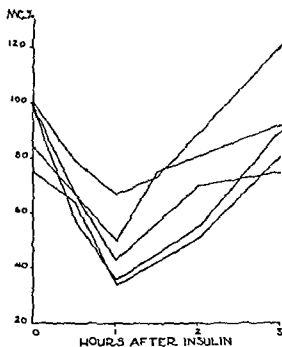
This observation has been confirmed by a series of insulin experiments made on 5 cats in which the spinal cord had been sectioned in the lower cervical region, the level of section varying from the roots of  $C_6$  to the caudal roots of  $C_8$ . The animals were operated upon under ether anesthesia, a segment of the cord was actually removed, and the dry operative field was carefully examined to assure removal of a complete section.\* Histologic examination of tissue from similarly operated cats has shown complete interruption of all fibers in every case.

Tests were performed three to five weeks after operation when all the cats were eating well and appeared to be in good physical condition. The dosage of insulin was 0.5 unit per kilogram body weight, injected intravenously, and the cats were fasted for fifteen hours before the tests.

Following this amount of intravenous insulin, blood sugar recovery took place normally. The average initial level was 91 mg. per cent; the average minimal level, 46 mg. per cent; and the average final level, 92 mg. per cent. The average initial and final levels compare well with those of the 7 normal intravenous tests of Graph 1, but the average minimal level was somewhat lower for the spinal cats (46 mg. per cent) than for the normal animals (55 mg. per cent). Two of the spinal cats showed hypoglycemic levels well within the normal range (67 mg. per cent, Cat K153; 50 mg. per cent, Cat K157), although the abnormally low levels of hypoglycemia of Cats K158 and K161 brought the general average 9 mg. per cent below that of normal cats. The significance of this difference is not known, although it may be attributed to disconnection of the thoracolumbar outflow of the autonomic system from the higher centers of medulla and hypothalamus. The ultimate blood sugar recovery was as good in the spinal as in the normal animals. Data from these tests are represented in Graph 2.

\*The animals were operated upon by Doctors Magoun and Clark, and were cared for by Dr. Clark.

Although external signs of hypoglycemia were not observed in any normal cats, such symptoms were marked in 3 of the 5 spinal animals. Coincident with the time of most marked hypoglycemia, the respiratory rate of Cat K153 rose to 300 per minute, of Cat K157 to 264 per minute, and of cat K158 to 360 per minute. Panting was vigorous and sustained, there was increased salivation, muscular flaccidity, and apathy to stimulation to such a degree that the state of the animals resembled mild coma. These symptoms were shown to a lesser degree by Cat K161, but Cat K155 appeared and reacted as normal throughout the course of the test, although its hypoglycemia was as marked as the others. The respiratory rate of K155 never rose above 28 per minute.



Graph 2—Blood sugar responses of spinal cats following intravenous insulin injection (0.5 u./kg. body weight).

There was no apparent correlation between the appearance of these external symptoms and the blood sugar reactions of the different cats, with respect either to the severity of the hypoglycemia or the amount of ultimate restoration. Spontaneous recovery from the symptoms took place as the blood sugar returned to normal levels.

#### DISCUSSION

Brooks<sup>6</sup> found that spinal animals were able to restore their blood sugar level to normal following doses of insulin as large as 0.5 unit per kilogram body weight given subcutaneously, whereas following larger doses many of the animals did not spontaneously recover. His work further indicated that the thoracolumbar outflow was capable of some functioning without connection with higher centers, since spinal animals in which the adrenals had been inactivated by removal of one and denervation of the other died from 0.5 unit per kilogram body weight dosage. Hypoglycemic symptoms which he observed included mewing, salivation, pupillary dilatation, and defecation; and "diaphragmatic" panting was frequently noted.

The data reported here confirm Brooks' work and compare directly the blood sugar changes induced by insulin in normal and spinal cats which were well recovered from the effects of the operation.

The insulin tests on spinal cats, however, furnish no explanation of the hypersensitivity to insulin which follows some hypothalamic lesions. With the same dose of insulin from which the spinal cats recovered, 4 cats with hypothalamic lesions showed marked symptoms of insulin shock, and 2 of the 4 (Cats A13 and A88) clearly showed prolonged and severe hypoglycemia. Ingram and Barris<sup>1</sup> observed hypoglycemic symptoms in cats with hypothalamic lesions following administration of a smaller dose of insulin, 0.5 unit total dose. Cleveland and Davis<sup>2</sup> likewise noted hypersensitivity to insulin using only 0.25 unit per kilogram body weight. Since spinal cats recovered normally from doses of insulin which produced dangerous reactions in certain cats with hypothalamic lesions, it seems unlikely that the hypersensitivity of the latter was due to destruction of any center normally acting by way of the thoracolumbar-sacral portions of the cord.

Another hypothesis has been suggested by the fact that the insulin hypersensitivity appeared in animals with experimental diabetes insipidus (see Ingram and Barris,<sup>1</sup> and Ingram and Winter<sup>3</sup>), which the work of this laboratory has shown to be due to atrophy of the pars nervosa of the hypophysis (Fisher, Ingram, and Ranson<sup>7</sup>). The antagonism between the action of insulin and posterior lobe extracts has been established by Burn,<sup>8</sup> Geiling, Britton and Calvery,<sup>10</sup> Houssay and Potick,<sup>11</sup> Magenta,<sup>12</sup> Houssay and Magenta,<sup>13</sup> and Ellsworth.<sup>14</sup> It seems reasonable to suppose that atrophy of the posterior lobe following hypothalamic lesions might leave the activity of insulin relatively unopposed. Our series of experiments, however, included cats which showed marked polyuria and polydipsia, yet which reacted normally to insulin, indicating that the diabetic symptoms and insulin hypersensitivity are not necessarily related.

A third possible explanation has been adopted by Ingram and Winter<sup>3</sup> who believe that the insulin hypersensitivity is due to suppression of the function of the anterior lobe; this supposition cannot at present be either proved or disproved. Insulin tests have been performed on monkeys in which the hypophyseal stalk was sectioned just proximal to the dorsal surface of the gland; but the results of the tests were normal (Brobeck, Magoun, and Ranson<sup>15</sup>). Interruption of fibers from the hypothalamus entering the infundibular process and pars distalis by way of the infundibular stalk, therefore, did not result in insulin hypersensitivity.

It seems probable that in some instances the abnormal sensitivity to insulin which appears to follow the production of hypothalamic lesions may be due simply to malnutrition, postoperative or respiratory infections, or inadvertent subcutaneous injection of a portion of an intravenous dose of insulin. No clear explanation has been found, however, for the hypersensitivity which was observed in 4 cats with diabetes insipidus and which has been reported by other investigators.

#### SUMMARY

Following subcutaneous injection of insulin in normal cats, hypoglycemia was more prolonged and in some instances more marked than that following intravenous injection of the same amount of insulin.

Normal fasting blood sugars and normal insulin reactions were noted in a large number of cats with lesions in various portions of the hypothalamus.

Insulin hypersensitivity was observed in 4 cats with lesions in the medial part of the anterior tuberal portion of the hypothalamus.

Spinal cats recovered as well as normal cats from 0.5 unit of insulin per kilogram body weight, injected intravenously.

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## ANIMAL TISSUE REACTION TO PARTICULATE COPPER STEARATE\*

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THE response of animal tissue to the introduction of particulate matter has been described principally with reference to silica or silicates in finely divided form. So characteristic a picture has resulted from contact with these materials both in experimental animals and in the human organism in certain occupations that the resulting syndrome has been distinguished by name from other pneumoconioses which present different or less characteristic clinical, roentgenologic, and pathologic features.

Those characteristics of silicosis which have warranted distinction from reactions to most other particulate matters are the cumulative nature of the tissue changes resulting from continued exposure, and their progression, even after contact with the causative agent has been interrupted. More or less distinctive tissue reactions have been considered relative to numerous other substances including barium,<sup>1</sup> carbon,<sup>2</sup> sulfur,<sup>3</sup> iron,<sup>4</sup> beryllium,<sup>5</sup> titanium oxide,<sup>16</sup> zinc,<sup>21</sup> and aluminum oxide.<sup>15</sup> Some organic dusts have been considered to lead to less typical and usually more acute reactions, such as shoddy fever and thresher's fever. With most such substances, however, the progressive and cumulative nature of the tissue reactions has been less notable than acute exudation or mere mechanical accumulation of the dust. In most instances in which important chronic tissue changes result from exposure to nonsiliceous material, they may be shown to be dependent upon contaminating silica or silicates, which independently exert their characteristic effects.

The possibility that copper in the form of the stearate when introduced in particulate form might give rise to a characteristic clinical and pathologic picture was suggested by the finding of marked pulmonary fibrosis in a patient whose history indicated heavy industrial exposure to copper stearate dust, and in whom no likelihood of inhalation of silica was apparent. Furthermore, the clinical and roentgenographic features of this patient were different in some respects from those usually encountered in silicosis.

### CASE HISTORY

A. P., a 46-year-old white male, was admitted to the Medical Service of Starling-Loving Hospital, complaining of dyspnea, dependent edema, and enlargement of the knees. His occupation had included farming and lumbering, and four years before admission for six months, the hauling of washed, wet river gravel and sand, the dust of

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which he insisted was negligible. He was well until two years before admission, when he was employed for nine days cleaning scale from a large copper vat, in which stearic acid had been prepared. The scale had dried and cleaning was accomplished by a motor-driven rotary wire brush. He used no mask and inhaled much dust, with the prompt development of a sore throat and cough. Edema of his feet and ankles soon appeared, with dyspnea on exertion which prevented work, though he remained ambulatory. These complaints became progressively severe with the production of a slight sputum that was repeatedly negative for tubercle bacilli. Within the past year curvature of the nails and clubbing of the fingers had appeared, with a slight soreness of the long extremity bones, and the loss of 20 pounds. Fatigability and precordial palpitation had become marked.



Fig. 1.—Roentgenogram of chest of patient presenting symptoms of pulmonary fibrosis and emphysema following intense exposure to copper stearate dust. Note reticulated fibrosis throughout all lobes of both lungs with emphysema.

On admission there was moderate cyanosis of the lips, with hyperpnea and dyspnea on slight activity. There was rounded prominence of the anterior tibial margins, and enlargement of the wrists, hands, ankles, and feet. The right axillary and both inguinal groups of lymph nodes were slightly enlarged, though discrete and soft. The heart was enlarged to percussion; sounds were distant, and there were no murmurs. The pulmonic second sound was moderately accentuated. Rhonchi were heard throughout both lungs, and coarse râles in both bases. Blood pressure was 110/72. The liver was palpable, but the abdomen was otherwise normal. Enlargement of and grating in both knees on motion suggested localized hypertrophic arthritis.

Biopsy upon a right axillary node revealed almost absent germinal centers, and slight hyperplasia of the reticulum; however, Hodgkin's disease could not be identified. The



chest roentgenogram (Fig. 1) showed a marked diffuse pulmonary fibrosis, suggestive of pneumoconiosis, but consisting in a fibrous reticulation rather than the nodular infiltration of typical silicosis. There was no area of pneumonic consolidation or of excavation. X-rays of the long bones showed the periosteal thickening of hypertrophic pulmonary osteoarthropathy. Serologic reactions for syphilis were negative, and the blood cytology and urinalysis were normal. The patient was discharged unimproved after a nine-day atelebric course.

#### LITERATURE AND MATERIALS

The toxicity of copper in the animal organism has been recognized, and has been held responsible for hemolysis and for development of a pigmentary hepatic cirrhosis. The possibility that absorption of the metal sufficient to induce such toxic results might follow the inhalation of particulate copper or copper-containing dust has also been pointed out.<sup>19</sup>

The development of a characteristic pulmonary clinical picture following the inhalation of finely divided copper or copper compounds was reported in 1887 by Lattimer<sup>13</sup> under the term "copperman's chest," a name applied by the laity to a disability among workers in copper smelters, particularly in the pulverizing and molding departments. The disease apparently consisted of bronchitis, emphysema, bronchiectasis, pulmonary congestion, and heart failure, with signs and symptoms of these entities. A brass-founders' ague was noted in some, but it may have been due to plumbism. A progressive pulmonary fibrosis was noted in all cases of the malady reported. The recognized contamination of the ores by iron, sulfur, tin, arsenic, and "earthy matters," however, rendered uncertain any conclusions regarding the pathogenicity of the copper itself and suggested the possibility of concomitant silicosis.

Murray in 1900 reported similar pulmonary changes with laryngitis, chest pain, dyspnea, and cough among workers in brass, which he preferred to attribute to copper rather than to lead.<sup>19</sup> Pneumoconiosis and a short working life were noted by Landis among the Butte, Montana, copper miners and smelters in 1916,<sup>12</sup> but were ascribed to silicosis. Hayhurst<sup>10</sup> and Hamilton<sup>9</sup> considered that of the "various afflictions" of copper miners "none can be charged to any peculiar toxicity of copper itself," and that "the ill health found among copper miners and workers . . . is caused by something else" such as lead, arsenic, free silica, carbon monoxide, or heat-fatigue toxins.

More recently pulmonary changes in copper workers have been reported by Waetgen<sup>23</sup> and by Arustamova,<sup>2</sup> but again not under circumstances eliminating the confusing possibilities of tuberculosis and silicosis. It is notable, however, that of the sixteen presumably tuberculous copper miners observed by Arustamova, no single instance was included of positive sputum or of pulmonary excavation. Such factors are compatible with a predominant role of silicosis in the production of the clinical picture. This is also indicated by the recovery of silica from the ashed lungs of these miners to an extent of 40.6 per cent of the insoluble residue; calcium and iron compounds were also found in notable percentages, but only a trace of copper could be demonstrated. Such reports are suggestive that the progressive and cumulative

effects of the inhalation of copper-containing dusts in the persons reported were due principally to the contaminating silica or silicates, but critical interpretations were not possible and animal experimentation was not made.

Accordingly, it was intended in the present experiments to determine the pathogenicity of copper compounds when introduced into animals in particulate form, especially in terms of their capacity to elicit tissue fibrosis and chronic foreign body reaction independently of contaminants. In view of the cited case history, copper stearate was employed for this purpose. The toxicity of this compound, used industrially in antifouling paints for the bottoms of vessels, has been recognized,<sup>6</sup> and its employment as a fungicide has been suggested.<sup>14</sup>

The copper stearate used was a fine green blue powder, light in weight, and graded by air flotation to particles with maximum diameters of 2 to 10 microns. These were fragments of monoclinic crystals, soluble in turpentine oil, chloroform, and benzol, but insoluble in water. Analysis showed a strong trace of iron, and an insignificant trace of silica, possibly resulting from the glassware used. Iodine number determination showed approximately a 3 per cent contamination with copper oleate. In the suspension of this material in saline for use in the present experiments sodium stearate was used in concentrations of approximately one part to one thousand of solution, to facilitate wetting the powder and to render the suspensions more permanent.

The copper stearate was introduced into guinea pigs by intraperitoneal injections and by dusting in a cloud chamber, as recommended by McCord<sup>17</sup> and by Haynes<sup>11</sup> for the determination of the pathogenicity of a finely divided substance. Such contacts have been shown to give rise to immediate irritative reactions to most highly insoluble particulate material regardless of its identity. Of the ultimate reactions, however, five general types are recognized by McCord:

- (1) Absorptive, with disappearance of the dust without reaction or scar tissue.
- (2) Inert, the dust remaining unchanged, without reaction.
- (3) Proliferative, with nodules of newly-formed fibrous tissue, increasing in size so long as the stimulating material is undissolved.
- (4) Mixed reactions with mixed dusts.
- (5) Miliary proliferation following mechanical dissemination of the substance by the acute exudative reaction.

A tissue response following exposures to copper stearate might be due to either the copper or the stearic acid radicals. The accumulation in guinea pigs of inhaled metallic copper dust has been shown by Remy<sup>20</sup> to be principally in the liver and spleen, and to a lesser extent in the lungs, without apparent local damage. "It could also be shown that within a few weeks . . . the increased copper was decreased, and the proper (normal) amount again appeared" on analysis of the individual tissues. The elimination from the blood within twenty-four hours of either ionic or colloidal forms of copper has been demonstrated by Yano,<sup>24</sup> with ultimate excretion of the metal in the bile rather than the urine.

The possible pathogenicity of the stearic acid radical was suggested by the identification of 10-methyl stearic acid among the tuberculolipids,<sup>22</sup> and by the specific tissue reactions to lipids noted by Fallon.<sup>4</sup> The unlikelihood of a specific tissue response to stearic acid was forecast, however, by the complete absence of a typical pulmonary reaction from the industrial medical files of a large soap and stearic acid manufacturer over a period of many years.<sup>14</sup> The condition of the present experiment, of course, permits evaluation of only the entire compound, copper stearate.

#### EXPERIMENT

To evaluate the pathogenicity of copper stearate in the present experiments, four series of animals were used. The first group of five guinea pigs received 100 mg. of copper stearate in saline suspension intraperitoneally through a gauge 20 needle introduced with local antisepsis through the rectus muscle. This dose was considered the first of a series of similar injections, but the entire group died within two hours after treatment, thus furnishing a measure of the immediate toxicity of the material and affording an opportunity to study the pathology of the acute poisoning. A second group of eight animals received from one to ten intraperitoneal injections at three- to four-day intervals of 25 or 50 mg. of copper stearate, and were sacrificed at from twenty minutes after the first injection to fifteen weeks after the last of eight injections. Upon death by chloroform anesthesia particular attention was paid to the omentum, the mesentery, and the abdominal wall in which some suspension was unavoidably deposited at each injection. The histopathology of these structures is reported below.

The third series of eight animals received a single heavy dusting of ten minutes' duration in a chamber with a motor-driven fan<sup>10</sup> and were sacrificed at intervals of thirty minutes to six months thereafter, thus permitting observations of the progressive or recessive character of the resulting tissue reactions. A fourth series of four animals was sacrificed at intervals during and after five months of frequent dustings, to permit the evaluation of the cumulative characteristics of the tissue reactions; three of these animals received 110 dustings each.

#### INTERPRETATION OF HISTOPATHOLOGY

In the interpretation of these tissue reactions it is important to recall the peculiarities of the guinea pig lung in which small areas of apparent congestion and of alveolar epithelial proliferation are fairly common, as also small lymph follicles about blood vessels, adjacent to bronchi, and scattered throughout the parenchyma, often infiltrated with eosinophiles. The recognition of intercurrent and of parasitic pulmonary disease is also important.

All animals injected intraperitoneally showed local reaction and absorption as well as reaction in the lymph nodes. Those which died early showed slight evidence of vasoparalysis and moderate liver damage; all had abscesses of the abdominal wall and fibrosis of the mesentery. The adrenal glands of the dead animals were depleted of lipoids, and the liver was deficient in glycogen. The lungs of some animals showed pneumonia of doubtful relationship to this experiment, and one animal showed pulmonary atelectasis

with extensive pneumonitis, which may have been an incidental process. Negative polymorphonuclear chemotaxis was suggested by the minimal numbers of these cells, even at the sites of abscess formation (Figs. 2 and 3).

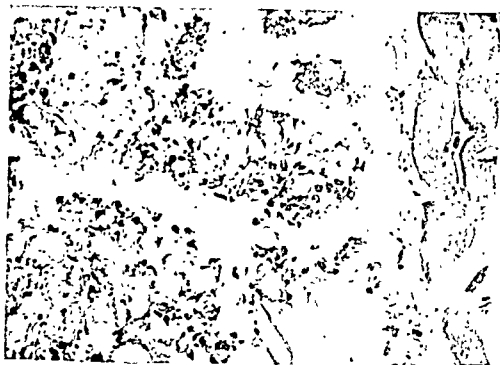


Fig. 2.—Section of abdominal wall of GP, dead after fifth daily injection of 5 mg. of copper stearate in suspension intraperitoneally and subcutaneously, showing presence of crystals with phlegmon and abscess formation. (Hematoxylin eosin  $\times 375$ )



Fig. 3.—Section of omentum showing presence of copper stearate crystals in fat tissues with slight leucocytic infiltration. GP died two hours after intraperitoneal injection of 100 mg. of copper stearate suspension. (Hematoxylin eosin  $\times 180$ .)

Those animals which survived and were killed by chloroform were remarkably well three months after the injections; the only pathology remaining was a slight fibrosis of the spleen and lymph nodes, with pigmentation of uncertain origin. There was no chronic liver or kidney damage. The local areas of acute reaction had apparently healed with pigmentation and slight fibrosis.

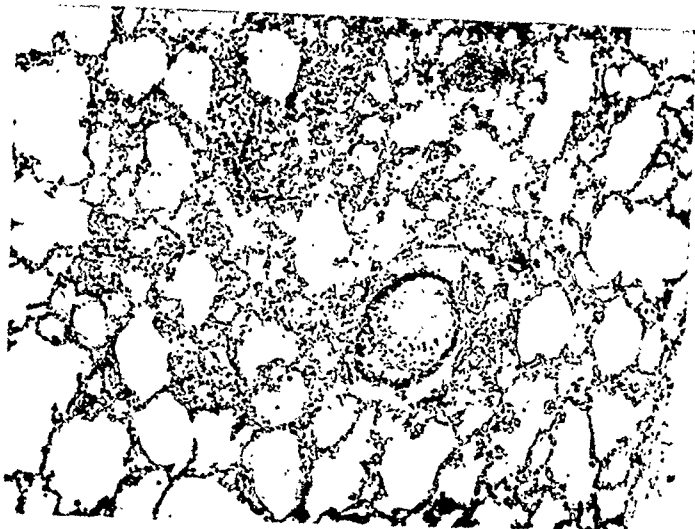


Fig. 4.—Section of lung of GP dead one day after single heavy dusting, showing leucocytic infiltration and edema. Desquamated epithelium and crystals fill the bronchus which is surrounded by atelectasis and compensatory emphysema. (Hematoxylin eosin  $\times 180$ .)

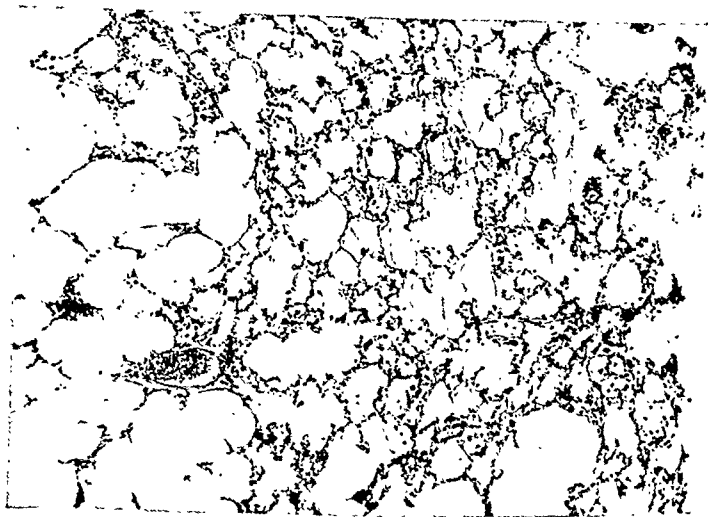


Fig. 5.—Section of lung of GP dead thirty minutes after single heavy dusting, showing acute pulmonary edema with leucocytic immigration and compensatory emphysema. (Hematoxylin eosin  $\times 180$ .)

The dusted animals revealed an acute injury to the mucosa of the bronchi, leading to desquamation and necrosis of epithelium, and exudation into the lungs. This apparently produced a partial atelectasis, with compensatory emphysema (Fig. 4). One animal died with acute pulmonary edema, suggesting anaphylactic shock (Fig. 5). At this period small areas of interstitial pneumonia could be found. If killed later the acute bronchial reaction had disappeared, but there remained irregularity of air distribution throughout the lungs.

If dusted repeatedly there was apparently not much cumulative effect. One animal died with intercurrent infection; the others showed a few scat-

tered nodules of histiocytes and leucocytes, and some undefined pigment in the pulmonary septa (Fig. 6). The bronchi were normal but there was some irregular subpleural infiltration. None of these changes could, however, be considered a severe impairment of lung function. The other organs were normal in the dusted animals except that the splenic pigment was often slightly increased and one animal showed a chronic infectious splenic tumor.

Thus all experiments both by injection and by dusting showed an acute necrotizing focal reaction, which had subsided within a month, and in no case was there evidence of progression or cumulation in the cellular reaction.

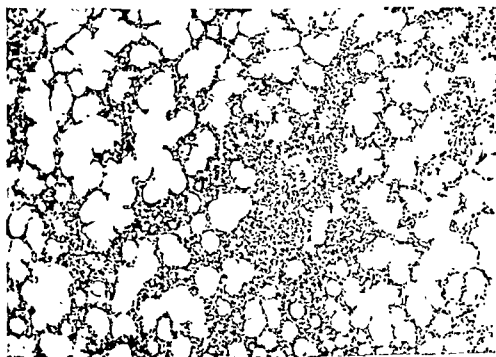


Fig. 6.—Section of lung of GP killed after numerous exposures over three months' time, showing nodular leucocytic and histiocytic infiltration and thickening of septa. (Hematoxylin eosin  $\times 180$ .)

#### DISCUSSION

Such observations would suggest the classification of copper stearate in the absorptive group of particulate materials as defined by McCord, with complete disappearance of the injected material after the acute reaction, without notable permanent change or scar tissue. This experiment, therefore, affords no support to an assumption that the reticulated pulmonary fibrosis of the clinical case reported here constituted a specific reaction to inhalation of copper stearate. The fundamental differences in structure of the lungs of guinea pigs and of man are recognized but appear of minor importance to the present experiment in view of the very similar reactions obtained in both to pathogenic particles of silica or silicate. The period of ten to fifteen minutes' heavy dusting to which the third group of animals was subjected, is by direct proportion equivalent to five to eight hours' dusting of the human animals, considering the relationships of their normal life spans; the greater intensity of dust encountered by the animals even further equalizes their exposures to that of the case reported, so that the present experiment would appear adequate to permit comparison of results.

## SUMMARY

1. The case is reviewed of a patient presenting marked diffuse pulmonary fibrosis of an unusual reticulated nature, under circumstances suggestive of pneumoconiotic origin, following the inhalation of copper stearate dust.

2. Several references in the literature recognize the incidence of pulmonary fibrosis among copper workers involving the variables of copper, tuberculosis, silicosis, and other mineral pneumoconioses.

3. Animal experiments were conducted to determine the tissue responses to copper stearate particles introduced subcutaneously, intraperitoneally, and by inhalation.

4. Such experiments demonstrate an acute reaction to copper stearate and its ultimate absorption and removal without residual or progressive chronic changes.

5. There has thus been afforded no experimental basis upon which to conclude that the pulmonary fibrosis noted in the patient was due to the copper stearate dust inhaled.

40 SOUTH THIRD STREET

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## THE DISTRIBUTION OF SULFANYL-2-AMINOPYRIDINE IN THE BODY\*

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DESPITE the widespread clinical use of sulfanyl-2-aminopyridine, very little is known of its fate in the body. That it readily penetrates all body tissues has been assumed on the basis of its therapeutic efficacy, but has never been demonstrated. In order to provide experimental verification of this assumption and to gain some information on its intermediate metabolism, the concentrations of sulfanyl-2-aminopyridine in the tissues and fluids of the dog have been determined at different intervals after administration of varying doses.

### EXPERIMENTAL.

Two-tenths gram of sulfanyl-2-aminopyridine† per kilogram body weight was suspended in approximately 25 to 30 c.c. of water and partially dissolved by the addition of several drops of concentrated hydrochloric acid. This solution was then administered by stomach tube to dogs under nembutal anesthesia. At specified time intervals from fifteen minutes to twenty-four hours, aqueous humor, blood, and cerebrospinal fluid were removed. The blood was obtained from the external jugular or femoral veins and the cerebrospinal fluid by suboccipital puncture. Only one sample of cerebrospinal fluid and two samples of aqueous humor could be obtained from each dog since the composition of these fluids is known to change after paracentesis.<sup>1</sup> At the conclusion of the experiment, the dog was killed by intravenous injection of ether, and portions of the various tissues were removed as rapidly as possible for analysis. Representative samples of approximately 1 Gm. were weighed and transferred to mortars, where they were thoroughly ground with sand and a minimum volume of 3 per cent trichloroacetic acid. This mixture was transferred quantitatively to a 15 c.c. centrifuge tube and allowed to stand overnight in the icebox. After centrifugation the clear supernatant fluid was decanted into test tubes calibrated at 10 c.c., and the precipitate twice washed with 2 c.c. portions of the trichloroacetic acid. The supernatant liquid and washings were diluted to the mark with more acid. This solution was diazotized and coupled with alpha dimethylnaphthylamine according to the method of Marshall and Litchfield,<sup>2</sup> observing the precautions of Stevens and Hughes.<sup>3</sup> When the solution was not perfectly clear, the turbidity was removed by filtration. Photometric‡ readings were

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†Generously supplied by Merck and Co., Inc., Rahway, N. Y.

‡Cenco—Sheard-Sanford "Photometer," using green filter No. 2.



then made to determine the concentration of sulfanilyl-2-aminopyridine. When plotted on semilogarithmic paper, the readings were found to be a straight line function of the concentration for all solutions containing less than 0.060 mg. Solutions more concentrated than this should be diluted to fall within the range. Blood was analyzed as described by Marshall and Litchfield,<sup>2</sup> except that 5 volumes of 15 per cent trichloroacetic acid were employed instead of 4 volumes advocated in their method. This slight alteration gave us water-clear filtrates, whereas with 4 volumes, the filtrates were faintly colored. The volume of saponin employed was correspondingly decreased. Aqueous humor and cerebrospinal fluid were similarly analyzed.

## RESULTS

As can be seen from Table I, sulfanilyl-2-aminopyridine has been found in

TABLE I

SULFANILYL-2-AMINOPYRIDINE CONCENTRATIONS\* AFTER LARGE AND THERAPEUTIC DOSES

TIME AFTER ADMINISTRATION	WITH LARGE DOSES									WITH THERA- PEUTIC DOSES
	MINUTES		HOURS							
	15	30	1	2	3	4	6	12	24	24
Blood	0.4	1.5	3.8	5.1	6.2	6.1	5.4	4.7	3.1	2.5
Spinal fluid		0.2	2.2	2.1	3.5	3.9	4.1	5.3	2.8	1.7
Aqueous humor	0.2	0.4	1.6	2.9	4.1	5.8	4.9	4.4	2.7	1.5
Vitreous humor		0.6	0.6	1.6	2.1	3.2	1.8	4.0	2.2	0.2
Sclera		0.7	2.4	5.0	6.2	7.0	4.1	11.5	2.9	0.8
Cornea		1.9	2.0	4.1	5.6	6.8	4.3	7.7	3.9	0.3
Lens		0.2	0.2	0.5	0.6	1.1	0.6	4.4	2.1	<0.1
Retina		0.6	5.7	6.3	4.6	6.3	4.4	11.5	3.4	1.8
Tendon			0.8	1.3		6.0	4.1	3.3	3.6	1.0
Bone marrow			0.9	1.3		6.1	4.9	4.2	2.5	0.2
Heart			1.6	2.9		5.3	2.7	2.7	2.0	0.1
Skin			0.8	3.1		6.8	3.9	8.7	4.6	1.4
Liver			4.7	4.9		9.2	4.1	10.7	2.4	0.9
Muscle			2.1	3.3		7.2	3.1	6.9	3.9	0.4
Fat			1.1	1.1		1.8	2.3	3.3	1.6	0.6
Lymph gland				5.8				4.5		
Spleen			3.5	4.5		6.6	4.1	7.1	3.0	0.8
Gastric wall			0.5	2.0		8.5	4.3	5.7	3.7	0.3
Gastric mucosa			17.9	30.9		27.3	45.5	12.9	2.9	0.8
Intestinal wall			3.1	1.6		7.0	5.1	6.7	3.9	0.3
Intestinal mucosa			5.9	1.3		6.0	2.4	2.1	5.0	1.3
Kidney			2.8	8.0		8.8	7.1	12.3	3.9	0.4
Pancreas			2.0	1.6		5.2	5.1	8.1	0.4	0.2
Adrenals			3.9	2.3		7.1	5.2	4.9	2.3	0.9
Brain				1.8		3.5	2.1	2.8	2.6	<0.1
Nerves			2.4	2.7		9.0	6.3	6.1	3.2	1.1
Lung			2.1	4.7		5.3	7.3	6.1	3.9	0.1
Trachea			1.0			6.1		2.8	1.8	
Thyroid			3.8	2.0		11.3	5.1	4.8	3.8	<0.1

\*Blood, cerebrospinal fluid, and aqueous humor are expressed as mg. per 100 c.c. All others are expressed as mg. per 100 Gm. moist tissue.

every tissue or fluid thus far analyzed. It was found in the blood within fifteen minutes after administration and attained its greatest concentration between the third and fourth hours. In the aqueous humor the values were somewhat lower, but the shape of the curve was similar to that of blood. However, the

penetration into the cerebrospinal fluid appeared to be somewhat slower since the maximum value was secured at the end of twelve hours, at which time it was actually higher than that of the blood. The lens showed the slowest rise of any tissue analyzed, an observation previously reported with sulfanilamide.<sup>4</sup> At the end of six hours, it contained only 0.6 mg. sulfanilyl-2-aminopyridine per 100 Gm. of lens. Most of the tissues followed the curve for blood and aqueous humor, attaining their highest concentration about the fourth hour. A number, however, did not reach a peak until the sixth to the twelfth hour. The concentration in the gastric mucosa was several times that of any other tissue. This was probably due to the mode of administration. Of the other tissues, the kidney, retina, sclera, and thyroid showed the largest amount of sulfanilyl-2-aminopyridine, and fat and the lipid rich brain contained the least.

In the experiment just described sulfanilyl-2-aminopyridine was employed in doses considerably higher than those recommended for clinical use. The effect of the drug administered in therapeutic doses was next investigated.

Dogs were placed under nembutal anesthesia, and 30 mg. sulfanilyl-2-aminopyridine per kilogram body weight were given by stomach tube. An additional 15 mg. per kilogram were administered every four hours for the duration of the experiment. Blood was removed after one and two hours and at the end of each four-hour period. Aqueous humor was aspirated after twelve and twenty-four hours, and cerebrospinal fluid and the various tissues were analyzed after twenty-four hours. The analyses were performed in the manner previously described.

The blood concentration remained quite constant for the period examined, ranging from 1.1 to 2.5 mg. per 100 c.c. from the second to the twenty-fourth hour. The aqueous humor and the cerebrospinal fluid contained slightly lower amounts (1.1 to 1.7 mg. per 100 c.c.) for this period. The values for the tissues are listed in Table I and range from a trace to 1.8 mg. per 100 Gm. of tissues.

#### DISCUSSION

It has already been shown<sup>4, 5</sup> that sulfanilamide readily penetrates into all tissues thus far examined. This fact has now been demonstrated for sulfanilyl-2-aminopyridine. As would be expected from its larger size, its penetrability into the cell is somewhat lower than that of sulfanilamide. Thus, the blood of dogs receiving 0.20 Gm. sulfanilamide per kilogram body weight contained 18.0 mg. per cent after four hours, whereas dogs receiving similar doses of sulfanilyl-2-aminopyridine contained only 6.1 mg. per cent. A similar diminution was noted in the other fluids and tissues analyzed. Despite this difference in the absolute content, the shape of the curves was very similar for both compounds. A greater variability was noted in the concentration of sulfanilyl-2-aminopyridine than sulfanilamide. Other workers<sup>6, 7</sup> have already called attention to this fact.

Considerably lower values were obtained when therapeutic doses were administered. These values were also lower than those secured on human beings receiving a proportional dose. Thus, the blood sulfanilyl-2-aminopyridine in our patients averaged 1.7 mg. per cent, whereas with patients similarly treated, the blood level was about 4 mg. per cent.<sup>8</sup> The blood sulfanilamide in dogs

receiving therapeutic doses has also been shown to be considerably lower than those obtained on man.<sup>4</sup> It seems probable that the amount of sulfanilyl-2-aminopyridine per unit weight required to maintain a definite blood level increases as the animal becomes smaller. However, it must be remembered that the superiority of a high blood concentration has not been definitely established. Thus Flippen and co-workers<sup>9</sup> found great variation in the concentration of sulfanilyl-2-aminopyridine among individuals receiving the same dosage, with no marked difference in chemotherapeutic effect. Further work is necessary to relate fluid and tissue concentrations with therapeutic efficacy.

### CONCLUSIONS

1. The method of Marshall and Litchfield for sulfanilamide determination has been modified to allow sulfanilyl-2-aminopyridine estimation in tissues.

2. The concentration of sulfanilyl-2-aminopyridine in various tissues and fluids of the dog has been determined one, two, three, four, six, twelve, and twenty-four hours after administration of 0.2 Gm. per kilogram body weight. The maximum concentration is reached about the fourth to the sixth hour.

3. The level in the blood and aqueous humor was determined when therapeutic dosage was employed.

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# LABORATORY METHODS

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## I. STUDIES OF LIVER FUNCTION IN HEALTH AND DISEASE\*

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### OBSERVATIONS ON A SIMPLE AND ACCURATE METHOD FOR QUANTITATIVE DETERMINATION OF THE BILE SALTS IN URINE AND BILE

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IT IS NOW one hundred and fifty years since the first attempts of Fourcroy and Vauquelin (1790) and later of Thenard (1807) and Berzelius (1809) to identify the various constituents of bile. Since that time there has not been a satisfactory method for the clinician in the practice of medicine to utilize the facts unearthed by innumerable investigators relating to the function of the liver in health and disease.

This communication presents the results of three years' studies in over 4,000 urine specimens and over 5,000 bile specimens on more than 500 patients over extended periods of time.

The method herein described is a stalagmometric one of estimating the surface tension of bile and urine, which gives the exact quantity of bile salts present. To date other tests for this purpose have presented certain noteworthy disadvantages.

(a) They have been elaborate, requiring the services of a skilled chemist, as with Doubilet's technique.

(b) They have been nonspecific for bile salts, as with the Pettenkofer reaction, originating in 1848 and still in use under numerous modifications. The Pettenkofer reaction has been shown to react positively with as many as forty other substances, by Thudicum (1868), Mylius (1887), Udranszky (1887), Tashiro and Mills, and Walker.

(c) Numerous tests, as those proposed by Chabrol, Charonnat, Cottet and Blonde, by Goiffon, Nepveux and Chaleil, Peoples, Etcheverry, Nakagawa and Fujikawa, Nakagawa and Yoshikawa, Sullman and Schaub, Borgatti (Giordano and Levi modification), and others, are unsatisfactory for clinical use due to uncertainty of color reactions, elaborate technique, great time consumption, expensive apparatus, or a combination of some of these impractical procedures. As an example, Minibeck's technique requires an elaborate "fluorescent microscopic spectrophotometer." The price of this instrument ranges in the neighborhood of \$800 to \$1000, and in addition must be designed and built individually.

(d) The Gregory and Pascoe reaction has been used extensively in recent years, depending upon a colorimetric reaction. However, as pointed out by Sobotka and especially by Doubilet, "it is specific for cholic acid alone and is

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of little value in the analysis of human bile which contains a large proportion of desoxycholic acid." At the maximum, cholic acid comprises 50 per cent of the total, as has been estimated by Doubilet and Colp, Sobotka, Weiland and Revery, and others, in human fistula bile from persons having had common duct obstruction. When inflammation of the ducts was noted at operation, Doubilet and Colp found cholic acid to comprise only one-sixth of the total bile acids. The error in relying on this variable cholic acid index as to the total amount of bile acids by the Gregory and Pascoe method is further seen when one finds that in addition to cholic acid, the other human bile acids are oxalic acid (Larvonat); desoxycholic acid, anthropolidesoxycholic acid, lithocholic acid, chenodesoxycholic acid (Weiland); cholalic acid (Schotten); fellic acid (Schotten); cholic acid and taurochenodesoxycholic acid (Weiland); and glycodesoxycholic acid (Hammarsten). Sobotka has likewise emphasized the wide variations cholic acid undergoes and its ratio to the other bile acids as soon as pathology sets in.

The surface tension method of estimating bile acids or salts in the urine has been investigated by Schemensky in 1920, and by Doumers, Gilbert, Chabrol, Benard, Mueller, Adlersberg, Meyer, Eppinger and Ranzi, Duco and Panza, and the essayists.

The Doumers first established the fact already known that bile salt solutions in distilled water in increasing concentrations lowered their surface tension following a regular and symmetrical curve to a certain limit (Doumer's law).

However, Duco and Panza found that this law is not applicable to urine or bile since electrolytes, as sodium or potassium salts, may cause considerable variations in the bile salt estimations (Schulze-Hardy law). This error was avoided by Duco and Panza's introduction of 1 per cent phosphoric acid, sufficient to bring the solution to the isoelectric point of  $pH^2$ . At this isoelectric point there is no alteration in bile salt solution by extraneous electrolytes or colloids. Hence urine or bile at a  $pH^2$  gives a surface tension figure which is specific for bile salts alone. When the solutions are at a dilution of less than 1:1,000, charcoal adsorbs practically 100 per cent of the bile salts. If then the surface tension of the bile or urine is taken at  $pH^2$ , and again taken after extractions with medicinal animal charcoal, the difference is accurately expressed in quantity of bile salts. Comparison is then made with a graph which gives the surface tension of increasing concentrations of pure sodium glycocholate in distilled water. This is shown for urine and bile in Figs. 1 and 2 in the stalagmometer used.

An ordinary stalagmometer is used to determine the number of drops of bile or urine by a hand counter.

We present a modified technique in lieu of that proposed by the foregoing investigators studying surface tension, and most recently by Duco and Panza, giving a simplified test with greater accuracy for clinical purposes. These modifications are as follows: (1) Considerable time is saved by omitting the extraction by medicinal animal charcoal. We found that if an accurate isoelectric point of  $pH^2$  in the bile or urine were obtained, for clinical purposes practically 100 per cent of the bile acids are obtained directly from the

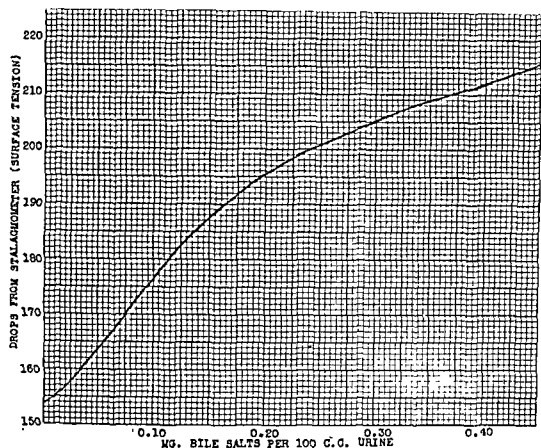


Fig. 1.

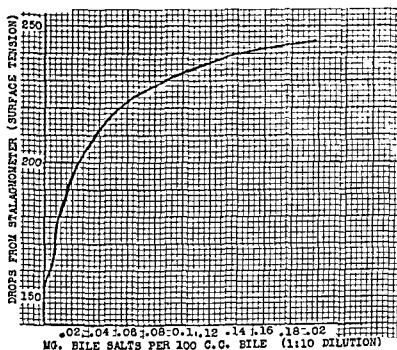


Fig. 2.

stalagmometer. Hence the charcoal treatment of the solution is unnecessary and time-consuming. (2) Instead of preparing a special solution of diluted phosphoric acid, concentrated hydrochloric acid is used for greater convenience and practicability. (3) The modification recommended by Duco and Panza, of arbitrarily adding 3 drops of the acid solution to the urine or bile to get the isoelectric point, is frequently inaccurate, particularly so in the urines. It is easily demonstrated that some urines or biles require several times this amount to get at the isoelectric point of  $pH^2$ . If this is not secured, the calculations, as advised by Duco and Panza themselves, may be very inaccurate, due to the variable contents of electrolytes and colloids occurring in different specimens examined. The  $pH$  of various specimens from the same individual is found to undergo wide variations at differing intervals.

The only accurate and dependable way to insure a pH<sup>2</sup> is to employ an indicator for this desired acidity. Tropeolin 00, as suggested by Dr. Robert Hamilton, is the best and most practical indicator for this desired pH. Hence a piece of filter paper dipped in the tropeolin 00 solution will turn pinkish red when the bile or urine being tested is at the correct pH<sup>2</sup>.

The surface tension of the urines and biles should be taken at about room temperature and at a rate of approximately 30 drops per minute. A stalagmometer can easily be made from a 15 c.c. pipette, by fusing a small-lumened glass to its end. The cost is approximately \$0.50 to \$1.00. A standardized stalagmometer can be bought from any appropriate chemical supply concern for a relatively small sum.\*

There are no normal or pathologic standards reported in the literature of the bile salt concentration in urine. Previous investigators have reported bile salt concentrations in the bile by methods described as inaccurate or impractical for clinical purposes. Hence in a separate communication these standards for bile and urine are established. It will be shown that the bile salt concentration in the bile is the most sensitive, direct, and accurate method of determining the physiologic and pathologic function of the liver. The value of bile salt estimations in the urine will also be reported.

#### SUMMARY

1. Observations on a simple, sensitive, and reliable method of determining the bile salt concentration in urine and bile are presented.
2. The bile salt concentration is determined by a surface tension method (stalagmometric).
3. The method requires no training or skill, and entails very little expense and time.
4. This method is most practical and accurate for determining the clinical significance of variations in the bile salt concentration of bile and urine. Clinical studies based on this technique follow in a subsequent report.

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\*This can also be purchased from the Precision Thermometer and Instrument Co., Broad and Brandywine Streets, Philadelphia.

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## THE FUCHSIN-FORMALDEHYDE METHOD OF STAINING ACID-FAST BACILLI IN PARAFFIN SECTIONS\*

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THE fuchsin-formaldehyde method, briefly mentioned in a previous discussion of the conditions affecting the staining of acid-fast bacilli,<sup>1</sup> has been used routinely in this laboratory for a year and a half, having been found to be the most reliable means of staining acid-fast bacilli in sections permanently, irrespective of the fixative employed. It has been found, however, that its usefulness depends upon the quality of the formaldehyde solution used.

The protocol of the method is as follows: washing in tap water after each step being understood:

### (1) Stain in

New fuchsin	0.5 Gm.
Phenol crystals	5.0 Gm.
Alcohol, methyl or ethyl	10.0 c.c.
Distilled water, to make	100.0 c.c.

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at 60° C. (paraffin oven) overnight for from twelve to twenty-four hours, or at room temperature for from twenty-four to forty-eight hours. The longer periods are recommended for *M. leprae*.

- (2) Freshly distilled aqueous formaldehyde, 5 to 30 per cent, five minutes.
- (3) Hydrochloric acid, 2 per cent, in 95 per cent alcohol, ten minutes.
- (4) Potassium permanganate, 1 per cent aqueous, until brown, usually two to five minutes.
- (5) Oxalic acid, 2 per cent, one minute.
- (6) Harris' hematoxylin, two minutes.
- (7) Van Gieson's stain, 3 minutes.

Acid fuchsin	0.1 Gm.
Picric acid	0.5 Gm.
Distilled water, to make	100.0 c.c.

- (8) Without washing, dehydrate in alcohol, clear in xylol, and mount in balsam.

Nuclei stain brown, connective tissue fibers red, muscle fibers yellow, and acid-fast bacilli dark ultramarine blue.

#### DISCUSSION

The preferred fixative is a mixture of equal parts of 10 per cent formaldehyde and 95 per cent ethyl alcohol. Fixation for several days, three to five or more, is noticeably superior to brief, twenty-four-hour fixation. Tissues preserved in this medium have given excellent results after preservation for several months.

The use of formaldehyde of the highest purity in the staining process is important. Aged or exposed solutions often give poor or no results, decolorizing the bacilli. The "reagent" grade of formaldehyde, preserved in dark bottles, usually is satisfactory, but any commercial formaldehyde, distilled through a simple water-cooled glass condenser, gives a usable product containing about 30 per cent formaldehyde. Solutions a few days old are satisfactory, but after three or four weeks, they may become very unreliable. It is advisable to use freshly distilled formaldehyde and to renew it for each batch of slides. Some dye is extracted by the formaldehyde, and the section is rendered a deep blue color.

The acid alcohol outwardly does very little, but seems to facilitate the subsequent bleaching process. The bacilli, rendered blue by the formaldehyde, will withstand vigorous treatment without being decolorized. The previous soaking in formaldehyde prevents much shrinkage which might otherwise take place during the bleaching. Tissues fixed without the use of heavy metals are readily totally decolorized, but a moderate residual pale blue color is largely masked by the hematoxylin.

By this method lepra bacilli, at times impossible to stain in paraffin sections by other means, have been found to be readily colored. The dark blue bacilli lend themselves very well to photomicrography, with purposeful understaining of the tissues.

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## A RAPID MEANS OF OBTAINING MANGANESE-FREE IRON\*

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MANGANESE-FREE iron is necessary in certain feeding experiments where manganese is to be excluded from the diet and where iron must be added. No iron salt or iron wire which we have tested is free from manganese, and there has, heretofore, been no rapid and convenient means of separating the two elements.

Reitzenstein<sup>1</sup> found that when finely pulverized ferrous chloride was vigorously shaken with pyridine and the mixture heated, a canary-colored crystalline mass appeared, which upon recrystallization from pyridine had the formula  $\text{FeCl}_2 \cdot 3\text{C}_5\text{H}_5\text{N} \cdot 2\text{H}_2\text{O}$ . This compound was unstable and rapidly changed to a basic salt. Pfeiffer<sup>2</sup> prepared an iron pyridine compound which he believed to be identical with that of Reitzenstein's, and which when exposed to the air, turned brown. Later, Moore and Miller<sup>3</sup> found that if pyridine is added to a solution of ferric chloride containing free hydrochloric acid, iron is precipitated quantitatively as the hydroxide. In all probability the brown-colored compound obtained by Pfeiffer was iron hydroxide. Moore and Miller also found that when pyridine is added to a neutral solution of a manganese salt in the cold, no precipitation of manganese takes place, but upon heating oxidation occurs and manganese comes down. They noted, however, that if the solution is made acid with hydrochloric acid, heat could then be applied without the precipitation of manganese. These observations furnished them with an effective means of separating the two elements. They precipitated ferric hydroxide by pyridine from a solution of ferric chloride containing hydrochloric acid, removed the solution by filtration, and washed the precipitate with water containing pyridine. The ferric hydroxide was dissolved in hydrochloric acid and reprecipitated by pyridine. After a number of reprecipitations they obtained manganese-free ferric hydroxide.

Orent and McCollum<sup>4</sup> prepared manganese-free iron by a modification of the Moore and Miller procedure, using filtration, in which they reprecipitated the iron as the hydroxide by pyridine eight times, and finally obtained a product containing less than 0.5 p. p. m.

By a further modification of the Moore and Miller procedure in which a special means of precipitation is used and in which centrifugation rather than filtration is resorted to, we have been able to prepare manganese-free ferric hydroxide from a solution of ferric nitrate containing 0.002 per cent manganese with one precipitation by pyridine, in which no trace of manganese can be detected. Iron wire containing as much as 0.2 per cent manganese,

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or ordinary ferric nitrate, ferric chloride, or ferric sulfate can be freed from manganese by this procedure with two precipitations.

#### PROCEDURE

Four grams of ferric nitrate are dissolved in 500 c.c. of water in a large beaker. To this solution, containing no hydrochloric acid, 7.5 c.c. of pyridine are dropped in from a 10 c.c. burette, a small drop at a time, while the contents of the beaker is rapidly revolved. Since the pyridine is added slowly and the solution thoroughly mixed, supersaturation takes place simultaneously throughout the entire volume of liquid, causing the hydroxide to appear in exceedingly small particles. This means of precipitation results in the bringing down, by occlusion, of a minimum amount of soluble manganese salt. An additional 0.5 c.c. of pyridine is run in and the hydroxide allowed to settle. The addition of more pyridine to the nearly colorless liquid causes no further precipitation. After removing most of the liquid by decantation, the contents of the beaker are transferred to two 50 c.c. centrifuge tubes. The tubes are rotated for not more than two minutes at a low rate of speed, after which as much of the remaining liquid as possible is poured off. The rotation must be slow and the time short, otherwise the hydroxide will pack, and packing makes washing ineffective. The precipitate, which is very soft and still contains a small amount of liquid, is washed into 500 c.c. of water containing 0.5 c.c. of pyridine. After vigorous rotation of the beaker to insure thorough mixing, the precipitate is allowed to settle and the water removed as previously. Five such washings are sufficient to free the hydroxide from manganese.

No hydrochloric acid is used in this procedure because precipitation of the hydroxide is brought about without heat, and manganese does not come down in the cold, even in the absence of hydrochloric acid. When a second precipitation is required, the residue is dissolved in hydrochloric acid, but just enough to bring it into solution. If acid is used in excess, more pyridine is required for precipitation.

#### SUMMARY

Removing soluble manganese salts, by filtration, from ferric hydroxide precipitated in the usual way is unsatisfactory. The precipitate brings down with it much manganese, and filtration causes the precipitate to form a gelatinous mass which makes washing slow and ineffective. Good results, however, can be accomplished by precipitating as already described, and by centrifugation. By regulating the time and the rate of speed of the tubes the washings can be removed by decantation, and the hydroxide kept in its original finely divided form. This makes for effective washing.

#### CONCLUSIONS

A procedure is described for removing soluble manganese salts from iron by a special means of precipitation, and by removing the washings by centrifugation rather than by filtration.

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## A NOTE ON THE PREPARATION OF HEMATOPORPHYRIN\*

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WITH the introduction of sulfanilamide and the occasional serious reaction that follows its administration, the detection of porphyrinuria, which sometimes accompanies these reactions, may become a procedure of routine laboratory analysis. Most of the methods for the detection of porphyrins are elaborate and require a familiarity with spectrophotometry.

In this laboratory it has been found possible to detect hematoporphyrin concentrations in aqueous solutions of one part in one million by examination for the brilliant red fluorescence of such solutions in "dark light" (the ultra-violet radiation of a water-cooled quartz Kromayer lamp from which visible light is filtered by a cobalt quartz screen). Before this procedure can be made available for general study, it will probably be necessary for hematoporphyrin to be available for the purpose of standardization. The material is not on the market in the pure form and even if it were, there would be some question as to whether the ordinary preparations represent true hematoporphyrin.

The majority of the methods for the preparation of hematoporphyrin from blood pigment depend on the use of heat and strong acids. It is now recognized, since the synthesis of porphyrins by Hans Fisher (1926) that many of the preparations of hematoporphyrin, by the older methods, are denatured, a fact reflected in the variability in absorption spectrum and solubility of different specimens.

The method of Laidlaw (1904) in which the iron is removed from ferroheme (reduced hematin) by the action of weak acid in the cold, is free from these objections. It has been found to be eminently satisfactory, gives an easily reduplicable product, and would seem to yield a hematoporphyrin that is close in its chemical structure to the nucleus as it exists in hemoglobin. In the original method the hemoglobin is kept in a state of reduction by treatment with hydrazine hydroxide and treated with 15 per cent hydrochloric acid which dissociates the pigment into globin and ferroheme, and then splits the iron from the latter. The final product is contaminated with a little protein that is difficult to remove. For this reason, the method has been modified by

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(1) precipitation of the corpuscular cholesterides, (2) digestion of the protein with pepsin, and (3) chloroform extraction of the porphyrin. Reduction is carried out by suction evacuation, which is continued throughout the process, since it has been found that in acid solution the reducing action of hydrazine is not manifest.

The yield by the present method is practically 100 per cent, though, since there are no expensive reagents, like hydrazine hydroxide, used, this factor would hardly enter into consideration as a reason for the adoption of the method.

#### PROCEDURE

Five hundred cubic centimeters of fresh, citrated, oxalated, or defibrinated blood (that of the dog, cow, pig, and human being has been found satisfactory) are allowed to stand in a 1 liter pyrex Erlenmeyer suction flask until the corpuscles have settled and the serum is removed with a suction pipette. One gram of U.S.P. pepsin is added, and after it has dissolved, 50 c.c. of ethyl ether and then 50 c.c. of toluene are added. The contents are shaken until they gelatinize. An excess of toluene may remain, but this should not be removed as it prevents frothing during the subsequent evacuation.

A one-holed rubber stopper which fits the neck of the flask snugly and through which has been placed the stem of a short, glass-stoppered separatory funnel so that the outlet is at least 3 inches from the fluid level when the stopper is inserted, is prepared. The side arm outlet of the flask is connected through a short length of rubber tubing, carrying a heavy screw clamp, to a T-tube which is connected with a vacuum pump. (A small water-trap type of pump is sufficient.) The free end of the T-tube is fitted with a rubber tube and pinch clamp.

With the stopcock of the separatory funnel closed and the glass stopper in place, suction is applied for one hour. Usually the reduction to hemoglobin will occur much sooner, but the suction is maintained to remove all free oxygen and ether, not only during the addition of the acid, but also for a considerable time thereafter.

About 100 c.c. of concentrated hydrochloric acid are measured into the funnel, and the glass stopper is replaced. The stopcock is turned cautiously and the acid is admitted to the flask, about 5 c.c. at a time, with constant gentle agitation. It will be noticed that wherever the acid comes into contact with the corpuscle paste, a thick greenish-brown rubbery coagulum is formed. From 70 to 90 c.c. of acid are required for complete coagulation. The glass stopper is left in place during the addition of the acid; it prevents its spilling during agitation of the flask. Should the stopcock be inadvertently opened too widely and all the acid aspirated, the preparation will not be ruined by the advent of air, which admitted at this stage will result in the formation of acid hematin. The best results are obtained when just sufficient acid is admitted to cause complete precipitation of all protein.

The clamp on the tubing connected with the side arm is closed, the vacuum broken at the T-tube, and the flask disconnected and transferred to an incubator. In about three hours the material is a liquid sludge. This is divided

between each of two 250 c.c. centrifuge bottles until the dark brownish material is packed and the supernatant toluene can be decanted. The bottles are then inverted to drain.

About 75 c.c. of chloroform are added to each sample, and the sludge is comminuted in it with a glass rod. On centrifuging the chloroform settles to the bottom, the coagulum rising to the top as a grayish mass of claylike consistency. This is perforated with the glass rod and the chloroform solution is decanted. It is not necessary to filter the chloroform extract; in fact, this procedure is attended by considerable loss of the solution.

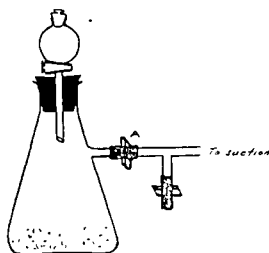


Fig. 1.

The chloroform solution from which the hematoporphyrin may be crystallized directly shows an intense red fluorescence as well as marked dichroism, being grass green by reflected light and purple red by transmitted light. On shaking with an equal volume of 2 per cent ammonium hydroxide, neutral amorphous hematoporphyrin is precipitated at the interface of the liquids. Stronger ammonia extracts the pigment from the chloroform as the ammonium salt, crystallizing as reddish brown rosettes on evaporation of the solution.

Neutralization of the ammonium hydroxide extract with dilute hydrochloric or acetic acid caused the precipitation of neutral hematoporphyrin. The latter was found to be so insoluble in water and in physiologic saline (less than 1:1,000,000 on the basis of the fluorescence test) that some question as to the reputed toxicity of *pure* hematoporphyrin arose. The same question has arisen repeatedly in connection with two other closely related substances, bilirubin and hematin, both of which are practically insoluble in the pure state at the pH of blood serum or tissue fluids, and for whose toxicity the weight of evidence appears to be in the negative. Insofar as ingestion by the water flea or contact with the human skin is concerned, the material prepared by the foregoing method seems to be devoid of toxic effects.

#### EXPERIMENTS WITH THE WATER FLEA

The technique described by Tinsley (1938) was followed. To a 500 c.c. culture of *Daphnia pulex*, 1 Gm. of amorphous hematoporphyrin was added. The animals survive indefinitely in the presence of the material. On microscopic examination the animals can be seen to continuously ingest and egest the pigment which passes through the entire alimentary tract without app

alteration. Examination of the culture water after twenty-four hours of such ingestion by a crowded culture of daphnia shows no solution of the pigment within the sensitivity of the fluorescence test.

#### EXPERIMENTS WITH HUMAN BEINGS

A 1 per cent solution of hematoporphyrin in acid alcohol was painted on an area 1 cm. square on the flexor surface of the forearm. After evaporation of the alcohol a brownish adherent film was left. This area was then irradiated with a carbon arc for ten minutes at a distance of 6 inches. Twenty-four hours later the pigment was removed with soap and water. The irradiated area, at this time, showed a moderate erythema except where protected by the hematoporphyrin film, where there had been no obvious change in the skin. Since this result indicated that hematoporphyrin could act as a screen against actinic rays, a film from a 1 per cent acid alcohol solution was painted over a small area of a lesion of psoriasiform lupus erythematosus on the forehead of a 40-year-old male and left in place for a week. There was no visible effect produced by this application as compared with similar unpainted areas in the same individual.

#### SUMMARY

A method for the preparation of hematoporphyrin is described. The material is practically insoluble in neutral aqueous solutions. It is not toxic by ingestion for *Daphnia pulex* and appears to be nonirritating to the human skin. Its solutions show a red fluorescence to ultraviolet light and the dried films act as a screen to that part of the spectrum which induces erythema of the skin following irradiation.

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## NITRITE FERRIHEMOCHROMOGEN AS A REAGENT FOR REDUCING SUGARS\*

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WHILE there are numerous specific chemical reactions whereby the presence of the reducing sugars may be detected and their quantities estimated, most of the routine laboratory reagents for this purpose depend upon the nonspecific reducing properties of these sugars toward various suitable oxidizing agents. Among these reagents in common use, we find in order of their frequency of utility, (1) alkaline cupric complexes, (2) ferricyanide solutions, (3) alkaline bismuth complexes, (4) alkaline picrate solutions, (5) alkaline silver or mercury complexes, and (6) solutions of redox potential indicator dyes, such as safranine, indigo, or methylene blue. All of these substances are capable of existing in a lower valence state, to which they may be brought by the action of any substance with a high reduction potential, such as dextrose at the boiling point, and in which state they exhibit physical or chemical differences from the oxidized form. In this sense, any of the reagents which depend upon the reducing powers of the sugar could hardly be made specific.

The alkaline metal complexes, however, have stood the test of time and laboratory utility. It is true that from a quantitative standpoint their reactions with the sugars have never been in true stoichiometric relationship, but there are several excellent quantitative methods which have been standardized empirically and are being improved constantly. Most of the improvement in the copper reagents, particularly, are designed to prevent the reoxidation of the reduced form which is to some extent inhibited by the relatively insoluble form of the latter in the case of the metal complexes, whereas in the case of the redox indicator dyes, it is so rapid as to almost preclude their use. In fact, the only reagents that are free from this objection are the alkaline picrates and ferricyanides, the high oxidation potential of which prevents their reoxidation by atmospheric oxygen; at the same time this potential makes them easily reduced by substances other than dextrose to the effect that results of quantitative determinations for dextrose made on biological fluids with these reagents are invariably too high.

The ideal reagent in this category would have a low redox potential, but the reductant would be removed from participation in the redox system so that reoxidation could not occur. The possibilities for such a reagent appeared from a consideration of the recently elucidated properties of nitrite-ferrihemochromogen.

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That "alkali hematin" solution was transformed to "reduce alkaline hematin" by heating with dextrose, has been known for over half a century. There seem to have been few attempts to utilize the reaction for the detection of dextrose because the reduced pigment rapidly reverts to the oxidized form. It later became recognized that pure hematin was irreducible by the reducing sugars in the absence of an adventitious "suitable" nitrogenous substance (hemochromogen former, Bertin-Sans and de Montessier, 1892) likewise present in its solution. It has been found that nitrite is such a hemochromogen former (Barnard, 1939) and that a solution of nitrite ferrihemochromogen, a greenish pigment, makes an excellent qualitative and semiquantitative reagent for reducing sugars, being converted by them to a scarlet pigment, nitric oxide ferrohemochromogen; the latter stable for several hours even in contact with atmospheric oxygen. The distinctiveness of the latter, as well as its characteristic absorption spectrum, which might make it applicable to a spectrophotometric method for the determination of these sugars, led to the development of a reagent which possesses several advantages over those commonly in use. The reagent is so flexible as to be equally applicable to the quantities of dextrose in the urine of a person with severe diabetes (4 per cent) or that in a 1:5 Folin-Wu filtrate of normal blood (0.02 per cent). It likewise is of quantitative significance, the time for reduction being in inverse proportion to the quantity of sugar present.

#### PREPARATION OF REAGENT

One hundred and sixty milligrams of crystalline ferriheme chloride (hemin\*) are dissolved in 1 liter of 5 per cent tribasic sodium orthophosphate containing 1 Gm. sodium nitrite. The reagent is ready to use upon solution of its constituents and keeps indefinitely. When kept in soft glass containers, there may be a precipitation of silicates, but this does not affect its utility and may be prevented by keeping the reagent in hard glass containers.

Since hemin is a dibasic acid, the phosphate is incorporated to buffer the solution and also to precipitate the alkaline earths where the reagent is used for the detection and estimation of dextrose in urine, otherwise insoluble magnesium and calcium salts of hematin are formed.

The reagent was found to be unaffected by creatinine, chloroform, urates, or polyphenols. From this standpoint, its usage in urinalysis was obvious. The application to blood dextrose determinations will be the subject of a further communication.

#### DETECTION AND QUANTITATIVE ESTIMATION OF REDUCING SUGAR IN URINE

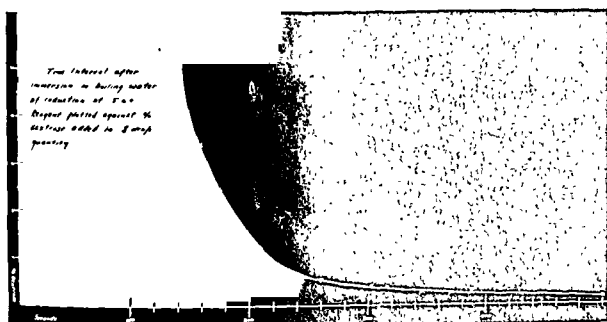
Five cubic centimeters of reagent and 8 drops of the urine to be tested are poured into an 8-inch by  $\frac{5}{8}$ -inch test tube. The contents are mixed and the tube is immersed in a boiling water bath, which should be of sufficiently large volume that it continues to boil during the immersion. An evaporating dish of porcelain, resting on the bottom of a pyrex beaker, makes a suitable

\*The hemin used in this study was prepared by the Pharmaceutical Division of the Armour Laboratories according to a modification of the original Chalfeff method (Barnard, 1932). Samples of hemin purchased from various other sources were found to contain extraneous nitrogen, so that, on alkaline solution, they would be reduced by dextrose without the necessity of the presence of nitrite.

water bath; the evaporating dish furnishes a background which facilitates reading the change in color on reduction of the reagent.

Reduction is signified by a rather sudden appearance of a scarlet color; the time interval of change from the greenish of the unreduced pigment is sufficiently short to permit a readable end point for the reaction.

Fig. 1 shows the interval from the time of immersion to that of reduction of various concentrations of dextrose. Where it is desired to conduct a quantitative determination on the basis of the addition of the test solution to the reagent at the boiling point, a graph constructed on this basis shows a shift of the entire curve to the left. This method of determination is not as convenient as that for which the graph is shown



In urines of normal reducing power, reduction will occur in from fifteen to twenty minutes of boiling. Five minutes has been the arbitrary time limit set upon this reagent, for if reduction does not occur within this time, the urine can be regarded as being without a quantity of reducing sugar of pathologic importance.

The reagent may be boiled directly over the free flame, as is the routine procedure for use with most of the qualitative copper reagents. Eight drops of urine are added to the boiling reagent, boiling is continued for one minute, and the mixture allowed to cool. The absence of reduction after a five-minute interval constitutes a negative reaction.

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## TOLERANCE OF DIABETIC PERSONS FOR DEXTROSE DURING VARIOUS TIMES OF THE DAY\*

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THE question of the tolerance of diabetic persons for dextrose during various times of the day is one of considerable scientific and practical importance. Although this problem has been studied by many students of diabetes, it can hardly be considered as conclusively settled. In this paper, we shall review and discuss the literature on the subject and present some experimental work.

The review of the literature may well be prefaced by the response to a query in which the editor of the *Journal of the American Medical Association*<sup>1</sup> answered in part as follows: "That the diabetic patient is at his worst in the morning, better at noon and best of all in the evening, is almost a proverb. In the morning his storage of carbohydrate is low and, as all agree, whether in diabetes or in health that is the time he utilizes carbohydrate least well; at noon and by the night meal necessary quantities of carbohydrate have been brought into action and are being metabolized and in consequence the entire machine is running more normally. Many a diabetic patient can take ten or twenty grams of carbohydrate on retiring and wake up sugar free."

The study of the blood sugar in diabetic persons during the day and night is of great importance. Pétren<sup>2</sup> was one of the first to observe the drop in concentration of blood sugar during the day in persons with diabetes. In 10 diabetic persons he found that the blood sugar one and one-half hours after the midday repast may even be lower than the "fasting level" at 8 A.M. He found further, that at certain hours of the day, the blood sugar may not rise at all after the ingestion of food containing starch. The most complete work on this subject is by Hattelhol,<sup>3</sup> who concluded as follows: During fasting the blood sugar curve does not exhibit a continuous decline, but is subject to oscillations, its fall from morning to evening being followed by a rise during the night (Fig. 1). This may be termed the paradoxical rise in blood sugar. It occurs in severe diabetics; not in the mild cases. It is not influenced by the character of the preceding diet, nor by food administered during the day. It is not due to muscular inactivity or the giving of meals during the night. It may be assumed that this phenomenon is due to sleep or some factor acting simultaneous with sleep. Vesa<sup>4</sup> has fully confirmed the findings of Hattelhol in the fasting diabetic person, and believes that it is not due to sleep but to an insulin-adrenalin mechanism. He contends, however, that the paradoxical rise is rendered indefinite by meals.

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Foster<sup>5</sup> found that in normal individuals a second dose of dextrose given one to one and one-half hours after the first caused little or no rise in blood sugar. Hamman and Hirschman<sup>6</sup> concluded from their experiments as follows: (1) The ingestion of dextrose in normal individuals in some way stimulates the mechanism of carbohydrate disposal so that the repeated ingestion of the same amount causes a less marked hyperglycemia. (2) The same stimulating effect is noted in diabetes; the second dose is followed by a less marked hyperglycemia and glycosuria. However, the difference between the effects of the two doses is less marked than in normal persons and varies in different stages of the disease. Possibly, when the diabetes is very severe, the difference may completely vanish. It should be noted that the second dose (each was usually 100 Gm.) was given from two and one-half to three hours after the first; that is, when the glycemie reaction from the first dose was declining or had just declined.

Several investigators had made similar observations previously. Most of them maintained that the fasting liver or pancreas is not ready for glycogen formation. They postulated that the hyperglycemia resulting from the first dose initiates the glycogen-forming mechanism which causes a fall in the blood sugar, and that this mechanism, not having subsided when the second dose is given, tends to prevent so marked a blood sugar concentration after that dose.

Jonas, Miller, and Teller<sup>7</sup> studied all-day blood sugar curves in nondiabetic and diabetic individuals receiving meals equally divided. They noted that the highest blood sugar and urine sugar occurred after breakfast, only when the interval between meals was short. They postulated that this may not be so when the interval is long. Sakaguchi and Sato<sup>8</sup> found that when identical meals were given three hours apart to normal individuals and mild diabetic persons, the second blood sugar curve was lower than the first. When the interval was six to eight hours, however, the blood sugar curves were the same. In more severe cases of diabetes, even when the interval was only three hours, the second blood sugar curve was not lower than the first. MacLean and de Wesselow<sup>9</sup> observed that when two doses of dextrose were given four hours apart to normal individuals, the blood sugar reaction to the second dose was independent of the first.

A decade ago Forsgren originated the concept that the liver works rhythmically in the performance of its various functions. Since then he has written extensively on this subject. He contends that the rhythmic function of the liver is not an isolated phenomenon, but an expression of rhythmicity of the entire vegetative system. This conception, as far as carbohydrate metabolism is concerned, Forsgren based on experiments he performed on rabbits.<sup>10</sup> He found that in these animals, glycogen accumulated during the night disappeared again the following morning. This phenomenon was entirely independent of food intake, and has been fully confirmed by Agren, Wilander, and Jorpes<sup>11</sup> who, as a result of extensive experiments performed on rats and mice, concluded as follows: There are cyclic changes in the glycogen content of the livers of rabbits, rats, and mice which are, to a large extent, independent of the intake

of food. Glycogen accumulates in the liver during the night and disappears to some extent during the next morning. Similar periodical changes occur, though to a minor extent, in muscles also.

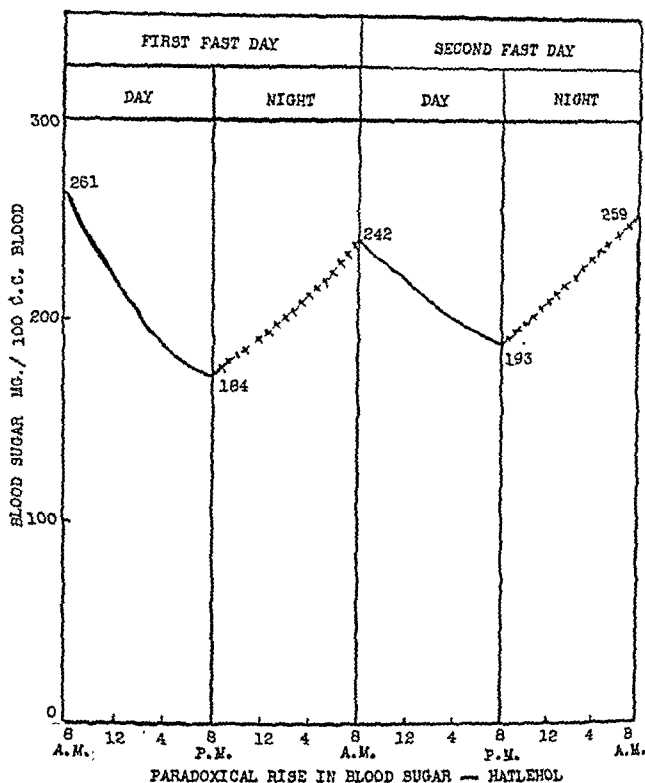


Fig. 1.

Chiefly with these findings as a basis, Forsgren has evolved the conception of the rhythmic function of the liver in the human being as far as glycogen metabolism is concerned. He contends that during the night and early morning hours the secretory or glycogenolytic phase occurs, which is associated with a rise in blood sugar and a decreased sensitivity to insulin. At noon the glycogenetic phase sets in; this is associated with a fall in blood sugar and an increased sensitivity to insulin, independent of meals. However, meals given during the glycogenolytic phase produce a greater rise in blood sugar than those given during the glycogenetic phase. In most individuals the glycogenetic phase occurs between 11 A.M. and 5 P.M., but may vary.

Möllerstrom<sup>12</sup> and Hopmann and Martini<sup>13</sup> are strong exponents of Forsgren's conception of the rhythmic function of the liver. Experimentally, the latter gave two 30 Gm. doses of dextrose to each of 6 diabetic persons. Both doses were given on the same day; one at 3 A.M., the other at 12 A.M. The blood sugar was studied from 3 A.M. to 7 A.M., and from 12 A.M. to 4 P.M. In 3 cases the blood sugar curves in the afternoon were identical with those in the morning; in the other 3 cases the blood sugar curves in the morning were higher.

An analysis of the literature leads one to conclude that the tolerance of persons with diabetes for dextrose during various times of the day can hardly be considered definitely settled. The following criticism may be offered of the experiments in which repeated doses of carbohydrate were given. In the first place, in the majority of cases the interval between doses was short, so that the second dose of dextrose was given when the glycemie reaction from the first was declining or had just declined. Furthermore, only a few cases were studied. Sakaguchi and Sato studied the reaction when the interval between doses was sufficiently long, but this was in very few instances. In the second place, the subjects all had mild diabetes. Conclusions drawn from results obtained from such cases are not applicable to persons with severe diabetes.

Forsgren's conception of the endogenous rhythmicity of the liver is based almost entirely on animal experiments in which the glycogen content of the liver was studied during various times of the day. It must be said that such evidence is insufficient for the establishment of a conception as to the function of the liver in the human being. Hopmann and Martini's experiments, in support of Forsgren's idea, are insufficient in number (only 6 cases were studied) and show inconclusive results.

#### METHOD AND RATIONALE OF PROCEDURE

Experiments were performed on 20 diabetic persons with varying degrees of severity. The subjects were all adult, ambulatory females. Only those taking unmodified insulin or no insulin were studied. Patients on protamine zinc insulin were excluded because of the prolonged action of the drug and the undesirability of discontinuing insulin for long periods of time. The effect of exogenous insulin was entirely eliminated by having those taking the unmodified insulin discontinue its use for from thirty-six to forty-eight hours before the beginning and during the entire period of experimentation.

To each of 20 persons with diabetes 70 Gm. of dextrose in a 15 per cent aqueous solution were given at 8 A.M. after a fast of fourteen hours. Specimens of blood (venous) were taken at the fasting level, immediately before ingestion, and one, two, and three hours after the ingestion of the dextrose. The sugar content of the blood was determined by the Polin-Wu method. Urine was collected one and one-half and three hours after the ingestion of dextrose, and its sugar content determined by the Benedict method.

Two days later, at 7 A.M., each patient received a meal consisting of two slices of bread, two eggs, and one orange. (The food value of this meal is carbohydrate 46 Gm., protein 18 Gm., and fat 12 Gm.) They did not receive anything else except water until 2 P.M., at which time they were given 70 Gm. of dextrose in a 15 per cent aqueous solution. The specimens of blood and urine were collected at the same periods as when the dextrose was given in the morning two days previously. In the interval between the tests the patients received their customary diets.

It will be noted that the second dose of dextrose was given seven hours after breakfast. It is reasonable to assume that the breakfast had been completely digested and absorbed, and that the glycemie and glycosurie reactions produced by it had long since subsided.

The question of the validity of comparing blood sugar curves of tests performed two days apart has been answered fully in several previous papers in which we used similar methods of experimentation. Briefly, it may be stated that dextrose tolerance tests, utilizing equivalent loads of carbohydrate performed on a series of diabetic persons two days apart at the same time in the morning, reveal identical glycemia and glycosuria.<sup>14</sup> Any differences in the blood sugar curves in the afternoon, as compared to those in the morning two days previously have, therefore, unqualified significance.

#### ANALYSIS OF RESULTS

Inspection of the concentration of blood sugar both before and after the ingestion of dextrose in the afternoon, as compared with that in the morning, revealed that persons with mild diabetes reacted in a manner strikingly different from those with the severe form. Accordingly, it became necessary to divide the cases into two groups. Purely arbitrarily, those with a fasting blood sugar below 200 mg. per 100 c.c. of blood were classed as mild or moderately severe cases, and those with a value above 200 mg. per 100 c.c. of blood as severe. The concentrations of blood sugar and amounts of dextrose in the urine before and after the ingestion of dextrose in the morning and in the afternoon, in persons with mild and moderately severe diabetes are recorded in Table I. In Table II similar data are recorded for the severe cases. The data in the tables will be analyzed under the following headings: 1. Concentration of blood sugar at the preingestion level. 2. Concentration of blood sugar after the ingestion of dextrose. 3. Urine sugar.

1. *Concentration of Blood Sugar at the Preingestion Level.*—In Table I (mild and moderately severe diabetes) it will be observed that the values in 8 out of 10 cases are somewhat higher at 8 A.M. than at 2 P.M. The mean difference is only + 7. The severe cases react in a strikingly different manner. In all the cases the values are decidedly lower at 2 P.M. and the mean difference is + 63.

2. *Concentration of Blood Sugar After the Ingestion of Dextrose.*—In the mild and moderately severe cases (Table I) the values two hours postcibal are higher in the afternoon in 8 out of 10 cases. The same holds true for 7 out of 10 cases at three hours postcibal. This is in contrast to the values at the preingestion level where the reverse is true; namely, in 8 out of 10 cases, the value is higher at 8 A.M.

In the severe cases (Table II) a distinctly different picture presents itself. In practically every case, at all levels, namely one, two, and three hours postcibal, as well as at the preingestion level, the concentrations of blood sugar in the afternoon are lower than the corresponding values in the morning. Thus the blood sugar curve in the afternoon, while it parallels that in the morning, is at a distinctly lower level.

3. *Urine Sugar.*—In persons with mild and moderately severe diabetes the urine sugar values were studied in only 7 cases (Table I). It will be observed that in 6 out of 7 cases the values are higher in the morning in the urine collected zero to ninety minutes postcibal. This may partly be explained by

TABLE I

CONCENTRATION OF DEXTROSE IN THE BLOOD AND QUANTITY OF DEXTROSE IN THE URINE OF PERSONS WITH MILD AND MODERATELY SEVERE DIABETIS IN THE MORNING AND IN THE AFTERNOON BEFORE AND AFTER THE INGESTION OF 70 GM. DEXTROSE

CASE NO.	BLOOD SUGAR (PER 100 C.C. BLOOD)								URINE SUGAR (GM.)			
	FASTING LEVEL		1 HOUR POSTCIBAL		2 HOURS POSTCIBAL		3 HOURS POSTCIBAL		0 TO 90 MIN. POSTCIBAL		90 MIN. TO 3 HOURS POSTCIBAL	
	8	2	9	3	10	4	11	5	A.M.	P.M.	A.M.	P.M.
	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.				
1	139	180	260	328	292	339	247	284				
2	153	127	248	231	258	263	230	211				
3	105	96	184	217	164	278	100	213				
4	156	143	306	274	328	345	274	284	2.1	0.0	1.5	0.0
5	182	124	304	292	318	305	204	183	1.4	0.7	1.6	1.7
6	165	139	308	367	312	308	247	217	1.6	1.0	2.4	0.7
7	196	188	350	312	356	381	246	274	5.1	1.9	6.4	3.6
8	148	132	282	218	305	325	237	274	1.7	0.8	5.1	4.2
9	127	109	274	254	252	282	179	228	1.5	0.7	1.1	1.3
10	96	160	286	339	325	392	263	305	1.2	1.6	3.4	4.3
Means	147	140	280	280	291	322	224	247	2.1	0.96	3.1	2.1
Difference between means*	+7		±0		-31		-24		+1.04		+1.0	

\*The sign is taken as plus when the value in the morning is greater and as minus when the value in the afternoon is greater.

TABLE II

CONCENTRATION OF DEXTROSE IN THE BLOOD AND QUANTITY OF DEXTROSE IN THE URINE OF PERSONS WITH SEVERE DIABETES IN THE MORNING AND IN THE AFTERNOON BEFORE AND AFTER THE INGESTION OF 70 GM. DEXTROSE

CASE NO.	BLOOD SUGAR (MG. PER 100 C.C. BLOOD)								URINE SUGAR (GM.)			
	FASTING LEVEL		1 HOUR POSTCIBAL		2 HOURS POSTCIBAL		3 HOURS POSTCIBAL		0 TO 90 MIN. POSTCIBAL		90 MIN. TO 3 HOURS POSTCIBAL	
	8	2	9	3	10	4	11	5	A.M.	P.M.	A.M.	P.M.
	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.				
1	312	245	455	364	513	408	409	342	12.3	6.4	25.2	16.9
2	216	196	390	342	460	377	351	288	1.7	1.4	14.9	3.1
3	350	298	392	360	420	410	384	330	8.4	7.1	12.3	9.6
4	242	151	378	294	400	328	262	206	7.0	4.4	9.6	4.9
5	208	133	400	351	312	276	216	183	4.2	2.0	2.0	1.5
6	203	179	392	381	385	388	298	306	5.8	6.2	8.7	12.2
7	235	138	449	305	540	367	476	303	0.5	0.5	3.0	1.8
8	237	160	385	348	449	408	342	312	5.4	1.6	2.3	6.2
9	267	216	364	348	412	392	315	310	4.7	4.5	8.8	6.4
10	261	180	430	435	435	440	345	339	8.8	5.3	7.0	4.9
Means	253	190	404	353	433	379	340	292	5.88	3.94	9.38	6.75
Difference between means*	+63		+51		+54		+48		+1.92		+2.63	

\*The sign is taken as plus when the value in the morning is greater and as minus when the value in the afternoon is greater.

the fact that the blood sugar is higher at 8 A. M. than at 2 P.M. in these cases. In 4 out of 7 cases the values are higher in the morning in the urines collected ninety minutes to three hours postcibal. In the persons with severe diabetes the values are higher in the morning in 8 out of 10 cases.



## COMMENT

The primary purpose of this work was to determine the tolerance of diabetic persons for dextrose during various times of the day. However, the method of procedure and the results obtained make possible the elucidation of various other related problems. The comment will, therefore, concern itself with a discussion of the relationship of the results to the following:

1. Tolerance of diabetic persons for dextrose during various times of the day.

2. Effect of repeated doses of dextrose on the blood sugar curve.

3. Forsgren's conception of the rhythmic function of the liver.

4. Hatlehol's so-called "paradoxical rise in blood sugar."

1. *Tolerance of Diabetic Persons for Dextrose During Various Times of the Day.*—Our work is limited to a study of the tolerance of diabetic persons for dextrose in the morning as compared with the afternoon. The prevailing conceptions are fully discussed in the review of the literature. The results of our experiments on 20 persons with diabetes of varying degrees of severity are sufficiently clear-cut to permit the drawing of categorical conclusions. The findings indicate that no general law applies, but that a definite difference exists between persons with mild and severe diabetes. In mild and moderately severe diabetes the tolerance for dextrose in the morning is equal to, if not greater than, that in the afternoon. In severe diabetes an entirely different picture presents itself. Here the tolerance in the afternoon is significantly greater than in the morning. The reason for this difference is in the present state of our knowledge inexplicable, and it would be futile to theorize as to the mechanism involved.

The prevailing impression is that the difference between mild and severe diabetes is purely a quantitative one. The difference in tolerance for dextrose in the afternoon as compared to the morning between the two groups is so marked, however, that the suggestion may be made that a qualitative difference is also present.

2. *Effect of Repeated Doses of Dextrose on the Blood Sugar Curve.*—Foster,<sup>5</sup> Hamman and Hirschman,<sup>6</sup> and Sakaguchi and Sato<sup>8</sup> found that in normal individuals and in persons with mild diabetes a second dose of dextrose produced a lower glycemic reaction than the first one. The explanation given is that the fasting liver or pancreas is not ready for glycogen formation. It was noted above that in their experiments the second dose was given when the glycemic reaction from the first dose was declining or had just declined. Maclean and de Wesselow,<sup>9</sup> in normal individuals, and Sakaguchi and Sato,<sup>8</sup> in diabetic persons, observed that when the interval was long, the glycemic reaction to the second dose was independent of the first one. In our series of persons with mild diabetes, the interval between breakfast and the ingestion of dextrose was seven hours. The glycemic reaction resulting from the breakfast had long since subsided. The blood sugar curve in the afternoon is at least equal to, if not higher than, the one obtained in the morning after the ingestion of an equal amount of dextrose. These findings are in complete accord with those of Maclean and de Wesselow, and Sakaguchi and Sato. The conclusion

to be drawn is that the capacity of the fasting liver (fourteen hours' fast) to store glycogen in the morning is no worse than in the afternoon, seven hours after the individual has had a breakfast containing a moderate amount of carbohydrate.

3. *Forsgren's Conception of the Rhythmic Function of the Liver.*—Briefly, Forsgren conceives as follows: 1. During the night and early morning, the secretory or glycogenolytic phase, which is associated with a rise in blood sugar and a decreased sensitivity to insulin, occurs. 2. At noon the glycogenetic phase sets in, and this is associated with a fall in blood sugar and an increased sensitivity to insulin. 3. Meals given during the glycogenolytic phase produce a greater rise in blood sugar than those given during the glycogenetic phase.

If Forsgren's conception is tenable, it should apply to all diabetic persons, and especially to persons with mild diabetes, since it is in this group that the capacity to store glycogen is greatest. Our findings indicate, however, that no general law applies, and that it is in the mild cases that the tolerance for dextrose in the morning is at least equal to, if not greater than in the afternoon. Accordingly, Forsgren's conception must be considered untenable in diabetic human beings.

4. *Hatlehol's So-Called Paradoxical Rise in Blood Sugar.*—As previously noted, Hatlehol found that the blood sugar falls decidedly during the day in severe diabetic persons, but only slightly in mild cases. He also noticed that this fall is uninfluenced by meals. A study of the preingestion blood sugar values (Tables I and II) at 8 A.M. and 2 P.M. fully confirms Hatlehol's findings.

#### CONCLUSIONS

1. The tolerance of diabetic persons for dextrose in the afternoon, as compared to the morning, was studied. It was found that no general law applies. In mild and moderately severe forms of diabetes the tolerance in the morning is equal to, if not greater than, that in the afternoon. In persons with severe diabetes, however, the tolerance is distinctly better in the afternoon.

2. The capacity of the liver to store glycogen in the morning, after a fast of fourteen hours, is no worse than in the afternoon, seven hours after a breakfast containing starch.

3. The findings made here make Forsgren's conception of the rhythmic function of the liver untenable as far as carbohydrate metabolism is concerned.

4. Hatlehol's findings of the paradoxical rise in blood sugar is in part confirmed.

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## DIFFICULTIES ENCOUNTERED IN A TEST FOR STANDARDIZATION OF TOXIN USED AGAINST SCARLET FEVER\*

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THE Dick reaction as a diagnostic method and as a guide to immunity has been accepted as a routine procedure by the attending staffs of hospitals for contagious diseases. However, certain anomalies of the test are known to exist. The discrepancies become particularly disturbing when they appear during the standardization of preparations used for testing and immunizing purposes. The source of the irregularities is not limited to one single factor. We are here presenting a limited group of observations in the hope that it may help to remove some of the complicating factors in standardization tests of preparations used for prevention of scarlet fever.

### HISTORICAL

In 1925 Joe,<sup>1</sup> in a comprehensive clinical study of the Dick test, observed the changing of a positive reaction to a negative, or vice versa, when readings were made eight hours and again twenty-four hours after injections. In the same year, Zoeller and Manoussakis reported a study of the influence on local immunity of repeated injections with one skin test dose. He pointed out that in some individuals a refractory condition of the skin developed, where the negative reaction was not associated with the development of general immunity. O'Brien<sup>2</sup> in 1930, reporting a careful study by Okell and Parish of "The Titration of Scarlet Fever Toxin," concluded that the difficult matching of two toxins would be possible only by a careful choice of subjects and by several readings of the reactions at different intervals after the injection. McGibbon<sup>3</sup> in 1934, from his observations on the Dick test in patients and convalescents, concluded that readings within the first sixteen hours would

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give a higher percentage of positives than readings made twenty-four hours after injections. Smiley<sup>6</sup> in 1935 suggested that inconsistencies were due to previous injections altering the response of the skin to subsequent skin tests. Ker<sup>6</sup> in 1937, in "Some Observations on the Dick Test" made in a study prompted by a run of anomalous reactions, stated that "the best interval to allow between performing and reading is probably between 12 and 18 hours." He also stressed the importance of the proper site for injecting. More recently, the Dicks<sup>7</sup> showed that 10 skin test doses injected intradermally produce in some individuals a local skin immunity regardless of their general susceptibility to scarlet fever.

#### OBSERVATIONS

For some time we have been experiencing difficulties while standardizing our testing and immunizing preparations on skins of institutional children. Much irregularity has been noticed, both in individual tests performed on a group of children on the same date and successive tests with the same preparations on different dates. In the first case, one preparation would appear much stronger than the standard on some children and weaker than the standard on others in a disproportionate manner. In the second case, a preparation would appear stronger than the standard on one date and weaker on another. The cause of these distressing facts was not understood, but was eventually suspected. As all the technical factors in these tests were carefully checked, we directed our attention to the medium on which the tests were performed, that is, the children. The possibility of a peculiarly modified local skin condition was considered. With this in mind, we planned the tests.

#### EXPERIMENTAL

##### I. Discrepancies in Simultaneous Reactions in Different Skin Areas of the Same Individual.

*Experiment 1.*—Two Dick preparations made up simultaneously in exactly the same manner in large gallon bottles were tested at the same time against the standard toxin on two groups of children, 15 in each group. Each toxin preparation to be tested and the standard Dick preparation were injected twice in each child. The results were surprisingly enlightening. One preparation was considerably stronger than the standard on one arm and weaker on the other; or the same preparation would give a stronger reaction on one arm than on the other, or would be completely negative on one and strongly positive on the other. The standard preparation itself gave different reactions in two different areas injected at the same time on the same individual. The range of variation was from strongly positive to weakly positive or from strongly positive to completely negative in about 50 per cent of all positive reactors. Protocol 1 of the first test illustrates this fact. A similar test was repeated with three other preparations following the same plan and the results were very much the same.

A circumstance should be pointed out that approximately coincided in time with the foregoing observation: the transfer of our testings to an institution where children spend many years and are subjected to repeated injections for diagnostic, immunizing, or investigative purposes (diphtheria, tuberculosis,

## PROTOCOL 1

## THREE PREPARATIONS OF DICK TOXIN EACH INJECTED SIMULTANEOUSLY ON BOTH ARMS

GROUP I	DICK PREP. NO. 88		DICK PREP. NO. 89		STANDARD DICK PREP. 1 S.T.D.*	
H. L.	16 × 20	12 × 15	11 × 10	-	-	13 × 15
C. T.	-	-	-	-	-	-
D. L.	-	-	-	-	-	-
J. M.	25 × 42	22 × 25	11 × 15	13 × 17	11 × 15	15 × 20
G. F.	-	10 × 12	-	-	-	9 × 9
S. S.	-	-	-	-	-	-
A. L.	10 × 16	15 × 16	15 × 15	15 × 18	-	20 × 20
A. M.	-	-	-	-	-	-
C. S.	16 × 19	20 × 19	16 × 17	20 × 30	9 × 10	20 × 16
F. Z.	15 × 20	-	12 × 14	12 × 15	-	11 × 14
A. S.	20 × 24	21 × 26	20 × 24	17 × 21	10 × 10	21 × 25
I. C.	-	-	-	-	-	-
R. N.	-	12 × 17	-	10 × 8	-	10 × 12
E. N.	-	-	-	-	-	-
M. Z.	-	-	-	-	-	-

GROUP II	DICK PREP. NO. 88		DICK PREP. NO. 89		STANDARD DICK PREP. 1 S.T.D.*	
J. G.†	-	-	-	-	-	-
F. B.	-	-	-	-	-	-
F. D.	10 × 12	-	12 × 12	12 × 11	12 × 11	-
B. F.	-	-	-	-	-	-
R. K.	15 × 20	9 × 10	14 × 14	15 × 15	10 × 9	20 × 20
M. S.	-	-	-	-	-	-
A. W.	-	-	-	-	-	-
W. G.	-	-	-	-	-	-
J. G.	14 × 20	12 × 12	-	11 × 14	10 × 10	-
R. H.	-	-	-	-	-	-
B. W.	-	-	-	-	-	-
J. G.	20 × 22	15 × 16	12 × 11	18 × 20	11 × 14	13 × 15
J. B.	12 × 15	-	-	10 × 10	-	-
T. M.	-	-	-	-	-	-
J. P.	-	-	-	-	-	-

Each double column represents the same preparation in corresponding areas of left and right arm.

\*Skin test dose.

†Reactions were not read.

- = No reaction.

etc.). Whether these result in a temporary state of local immunity to the streptococcal toxin or, on the contrary, in increased local sensitivity (specific or nonspecific), we leave for subsequent discussion. Here we shall only mention that either of these induced conditions might be the cause of the discrepancies under discussion.

## II. Discrepancies in Results in Two Successive Readings at Eight Hours and Twenty-Two Hours After Injection.

The possibility of the existence of another factor creating the described discrepancies, a factor that may add to the distorting influence of repeated injections, occurred to us for the following reason: Some normal children, such as those in private practice, where no repeated injections are likely to have taken place, sometimes give irregular and contradictory skin test readings. In view of the peculiarly early appearance of the positive reactions to the hemolytic streptococcus toxin, it seems possible that in some individuals an early appearance of a positive reaction is followed by its early disappearance and is thus missed at the established time of reading—within 24 hours. With this possibility in view, another experiment was carried out (Protocol 2).

*Experiment 2.*—Forty-three individuals, most of them known to be positive reactors, were inoculated with a total of 258 injections (3 injections on each arm of every individual) at 10 to 11 A.M. The results were observed and measured twice: within 7 to 8 hours and again the next morning—within 21 to 22 hours after the injections were made. The comparison of the two series of readings confirmed the supposition that time was a factor in the irregularities observed. The results were as follows: 43 (Table I, first test) individuals received 6 injections each; of these, 23 individuals gave negative reactions to all 6 injections in 7 to 8 hours, and remained negative during the second reading in 21 to 23 hours; 10 individuals gave all positive reactions to the 6 injections and these too remained unchanged in 21 to 23 hours. This gives a total of 33 individuals (out of 43) with unchanged reactions for two readings—whether positive or negative. However, the remaining 10 individuals (out of 43) gave reactions that reversed between the two readings, these reversals working both ways. Two individuals changed reaction from negative in 7 to 8 hours to positive in 21 to 22 hours, and 8 individuals reversed reactions in the opposite direction, being positive at the first reading in 7 to 8 hours and becoming completely negative at the time of the second reading in 21 to 22 hours. (The change of reactions in an individual affects one injection or more than one, and occasionally all 6.)

TABLE I  
SUMMARY OF 4 TESTS

NUMBER OF TEST	NUMBER OF INDIVIDUALS IN EACH TEST	NUMBER OF INDIVIDUALS WITH ALL REACTIONS UNCHANGED IN BOTH READINGS				NUMBER OF INDIVIDUALS WITH CHANGED REACTIONS IN AT LEAST ONE INJECTION			
		ALL NEGATIVE	ALL POSITIVE	TOTAL UN-CHANGED	PER CENT	- TO +	+ TO -	TOTAL CHANGED	PER CENT
1st	43	23	10	33		2	8	10	
2nd	33	26	3	29		2	2	4	
3rd	37	26	0	26		2	9	11	
4th	47	33	6	39		8	0	8	
Total	160	108	19	127	79.5	14 (8.75%)	19 (11.9%)	33	20.6

Three additional similar tests were performed with double readings, each test representing a different type of result: Test 2 with an even proportion of "positive-to-negative" and "negative-to-positive" changes; Test 3 with a predominance of the "positive-to-negative" change (as in the first test analyzed in detail); and finally, Test 4, in which, on the contrary, an increased number of positive reactions were observed during the second reading—with none in this group of 47 individuals showing an early positive reaction changing to a negative the next morning.

Thus the four experiments represent apparently all types of constancy and changeability between readings taken at two different time intervals. The summary in Table I brings out the following: In four tests with a total of 160 individuals, 127 persons, or 79.5 per cent, gave "stable" reactions, either positive or negative, during both readings. The remaining 33 individuals, or 20.6 per cent, with changing reactions gave the following distribu-

## PROTOCOL 2

Three Preparations of Dick Toxin Each Injected Simultaneously on Both Arms, With Reactions Observed in 8 and 24 Hours After Injections

	DICK PREP. NO. 195		DICK PREP. NO. 198		STANDARD DICK PREP. 1 S.T.D.*	
W. S.	18 × 15++ 12 × 14±	18 × 25 13 × 20	20 × 15++ 8 × 12±	18 × 25++ 15 × 20+	18 × 15++ 15 × 16+	18 × 25++ 17 × 20±
A. H.	13 × 16 Scratched	8 × 10± —	8 × 8 —	8 × 8 —	— —	— —
I. B.	10 × 8± 10 × 15	14 × 15± —	10 × 10 —	8 × 10 —	— —	— —
N. G.	10 × 20 15 × 24	14 × 20 14 × 23	20 × 16+ 16 × 21±	8 × 10 10 × 15±	12 × 12 10 × 12	14 × 20 17 × 20++
A. P.	— —	9 × 10 —	— —	8 × 8 —	— —	— —
S. S.	— —	— —	— —	9 × 12 —	— —	— —
H. R.	18 × 25++ 20 × 28	15 × 28 15 × 15±	18 × 25++ 20 × 17++	15 × 30++ 16 × 20±	20 × 23++ 15 × 20±	18 × 28++ 16 × 24++
E. M.	9 × 9 —	— —	— —	8 × 8 —	— —	— —
R. R.	— —	10 × 15 —	— 7 × 7	— —	— —	— —
R. C.	— —	— —	— —	— —	— —	— —
G. R.	23 × 30 —	18 × 20 15 × 18	25 × 25 18 × 21	20 × 25 20 × 20	25 × 25 12 × 12	25 × 28 20 × 25
R. H.	— 19 × 30	15 × 25 20 × 28	15 × 16 18 × 28	12 × 15 16 × 24	14 × 12 18 × 21	10 × 15 19 × 26
R. V.	— —	8 × 8 —	— —	8 × 8 —	— —	— —
J. G.	— —	7 × 10 8 × 8	— —	8 × 8 —	— —	— —
A. S.	20 × 30++ 20 × 25+	20 × 28++ 20 × 26±	20 × 30++ 20 × 25+	20 × 28++ 18 × 24±	25 × 25 18 × 20	22 × 30++ 20 × 20±
W. S.	10 × 10 —	6 × 9 —	— —	6 × 8 —	— —	— —
N. S.	15 × 15 18 × 18	22 × 30 —	14 × 12 —	20 × 26 15 × 15±	25 × 28 —	23 × 23 10 × 12±
W. M.	— —	— —	— —	— —	— —	— —
C. M.	20 × 30 20 × 30	18 × 25 19 × 25	8 × 8 —	15 × 25 19 × 24	9 × 11 9 × 11	15 × 17 15 × 16
E. V.	17 × 25 15 × 25	15 × 35 17 × 28	12 × 15++ —	20 × 30 15 × 25	20 × 30 22 × 28	20 × 25 19 × 25
R. S.	18 × 25 18 × 20	24 × 25 19 × 20	15 × 25 18 × 25	14 × 20 15 × 18±	15 × 15++ ±	20 × 20 18 × 18
T. L.	— —	— —	— —	— —	— —	— —
J. M.	— —	— —	— —	— —	— —	— —
W. B.	8 × 8 —	— —	— —	— —	— —	— —
G. R.	12 × 12 —	— —	8 × 8 —	— —	— —	— —
W. H.	— —	— —	— —	— —	— —	— —
R. D.	25 × 32 25 × 25	20 × 30++ 20 × 30+	22 × 28 20 × 27	20 × 28++ 25 × 30±	18 × 20 18 × 18	22 × 25++ 25 × 25±

## PROTOCOL 2—CONT'D

	DICK PREP. NO. 195		DICK PREP. NO. 198		STANDARD DICK PREP. 1 S.T.D.*	
R. T.	18 x 28 15 x 22	18 x 30+ 18 x 25±	16 x 26 15 x 20	18 x 25+ 16 x 20±	15 x 18+ 14 x 15±	15 x 20+ 15 x 19±
L. S.	20 x 22+ 17 x 19	18 x 25+ 18 x 25±	20 x 25+ 12 x 18±	20 x 25+ 18 x 18±	16 x 20+ 15 x 16±	20 x 25+ 18 x 23±
W. S.	23 x 22 26 x 30	15 x 15+ 15 x 15±	15 x 20+ 10 x 12±	15 x 15+ -	10 x 10 10 x 14±	15 x 15 11 x 14±
S. M.	7 x 7 -	- -	6 x 6 -	± -	9 x 10 -	± -
V. M.	20 x 23+ 20 x 26+	20 x 27+ -	20 x 26+ 20 x 28+	25 x 30+ 25 x 28±	20 x 22+ 20 x 22+	20 x 25+ 25 x 30+
S. G.	- -	- -	- -	- -	- -	- -
S. W.	- -	- -	- -	- -	- -	- -
E. B.	17 x 22 23 x 24+	10 x 20 16 x 20	20 x 24 18 x 20	18 x 29 20 x 24	15 x 18 17 x 20	23 x 20 25 x 25
S. S.	8 x 8 15 x 15	- -	- 10 x 14	- 11 x 13	9 x 10 13 x 19	- 12 x 12
M. M.	- -	8 x 8 -	8 x 10 -	- -	6 x 8 -	- -
B. S.	22 x 27 22 x 28	16 x 25+ 16 x 22±	16 x 29+ 17 x 25+	20 x 35+ 20 x 30+	18 x 23 18 x 22	18 x 19 20 x 22
R. C.	- -	- -	- -	- -	- -	- -
V. H.	- -	- -	7 x 7 -	- -	7 x 7 -	- -
B. Br.	- -	7 x 7 -	- -	8 x 8 -	8 x 8 -	- -
A. J.	15 x 15 -	18 x 18 -	15 x 15 -	19 x 20 -	17 x 15 -	17 x 17 -
A. L.	- -	- -	- -	- -	- -	- -

Each double column represents simultaneous injections with the same material in corresponding areas of left and right arm.

\*Upper and lower results on each line represent the 8 hours', and 21 to 22 hours' readings, respectively.

\*Skin test dose.

tion: 14, or 8.75 per cent, were negative at the end of the first day, turning to positive the next morning; 19, or 11.9 per cent, showed the opposite positive during the early reading (7 to 8 hours) and disappearing to totally negative at the last reading in 21 to 22 hours. The 11.9 per cent includes cases with reactions that changed from positive to negative; in some of them only one reaction changed; in others, 6 reactions changed, while in most of them, the number of positive-to-negative reversals was between these two extremes. Considering that in an ordinary Dick test any one of these six sites might have been selected for the injection, even the cases in which there is only one change of reaction should be considered significant.

## DISCUSSION

The observed anomalies in a toxin-standardization test bear an analogy to certain peculiar phenomena occurring on the skin during an acute disease—whether caused by *Streptococcus hemolyticus* or by an unrelated infection.

In 1925 Zingher\* described observations in scarlet fever patients in whom the site of a previous Dick positive reaction appeared entirely blanched out



amid the surrounding intensely red scarlatinal rash. He ascribed this to local cellular immunity caused by a previous injection of the Dick testing dose. He also gave the same interpretation to a similar observation on scarlet fever patients by Moriwaki.<sup>9</sup> On the other hand, evidence of an opposite nature has been seen in patients with either scarlet fever or acute diseases of a different origin than *Streptococcus hemolyticus*, such as staphylococcus or measles. It was brought out by Ferry,<sup>10</sup> Toomey,<sup>11</sup> and Moriwaki<sup>9</sup> that the site of a previous Dick reaction becomes greatly intensified in color during a subsequent attack of scarlet fever, measles, or acute staphylococcus infection. These authors agreed with Toomey<sup>11</sup> that the observed phenomenon was caused by a nonspecific sensitization of the skin in a previous Dick test. Confirming these two opposite types of observations, Goodall<sup>12</sup> in 1936 presented an interesting case, which combined the two features described: During an attack of scarlet fever one previous site of a Dick reaction was blanched out, while another (of an earlier date) on the same patient reappeared as a positive reaction sharply separated from the scarlet fever rash by its intensified crimson red color.

The described phenomena refer to observations of a site of an old Dick test during a subsequent illness caused either by *Streptococcus hemolyticus* or by different infection (staphylococcus, measles). However, these observations seem to throw light on the paradoxical results in a Dick test itself. If a previous injection with one skin test dose is sufficient to prevent the appearance of rash in the presence of the circulating toxin in a scarlet fever patient, how much more chance there must be for the prevention of a Dick positive reaction in a nonimmune healthy person when only one skin test dose of testing toxin is subsequently injected. On the other hand, an intensification of a previous Dick reaction in the presence of measles or a staphylococcus infection suggests nonspecific sensitization analogous to a positive reaction in a generally immune healthy person, caused by previous injections with unrelated antigens. The evidence in our own experience points to the same cause. Repeated injections for diagnostic and immunizing purposes with different antigens used in a large institution for diagnostic and immunizing purposes modify the normal sensitivity of the skin in two possible ways: some areas (in certain children) become refractive to subsequent injections with the Dick material, causing false negative reactions; others become unduly responsive, causing false positive reactions.

With regard to variations of results produced by an early fading of a positive reaction, the reason is not very clear. There is a possibility that the same factors causing pseudonegative or pseudopositive reactions will result in an early fading of a positive reaction—if either the local cellular immunization of the skin by a previous injection was incomplete or its local sensitization by a nonspecific substance was incomplete. In either case a reaction of brief duration is conceivable.

#### SUMMARY AND CONCLUSIONS

1. Irregularities in skin reactions have caused difficulties in standardization of scarlet fever toxin preparations.

2. Evidence presented in this paper and in works previously published points to repeated injections as a factor in an altered reactivity of the skin.

3. The following safeguards against difficulties in standardization tests in institutions where repeated injections are practiced are suggested: Two injections of each unknown preparation should be made against two injections of the standard toxin in each individual in a test. Therefore, only two toxin preparations could be standardized in a single test with a group of individuals, instead of the five preparations ordinarily tested

4. Two readings of results—in 7 to 8 hours and in 21 to 22 hours—are preferable when convenient and practical. A more suitable interval between injections and single readings might be worked out through a greater number of observations.

We wish to acknowledge our indebtedness to Dr. Morris Siegel and Dr. I. J. Brightman for their very helpful cooperation in the matter of arranging for tests, and especially for the precision and care with which the injections were made.

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## MODIFICATION OF HUDDLESON'S OPSONOCYTOPHAGIC REACTION\*

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THE opsonocytophagic reaction, introduced in 1933 by Huddleson and his co-workers,<sup>1</sup> has assumed a position of considerable importance in the diagnosis of brucellosis and in gauging results of treatment. However, the technique as originally described is difficult, in that scrupulously clean glassware is essential, the examination of leucopenic bloods is time-consuming in thin preparations, and there is danger of infection if the two cover-slip method of spreading the blood is employed. These difficulties are largely eliminated by the following modification.

\*From the Clayton Foundation for Research, Petroleum Bldg., Houston, Texas.  
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*Cleaning of Glassware.*—The glass slides may be cleaned satisfactorily by washing well in soap and hot water. Sodium hexametaphosphate (sold by numerous scientific supply houses under the trade name of Calgon and Calgonite) is an aid if the water is hard. After drying, the slides are washed in automobile antifreeze solution (such as Du Pont's "Five Star"), which is an inexpensive excellent grease solvent, and polished with a clean linen cloth.

*Preparation of Spreads.*—After incubation of the mixture of blood and organisms as described by Huddleson, several large drops of cell sediment are pipetted from the bottom of the tube, placed on one end of a slide, and spread in the usual manner by dragging the blood with another slide held at an angle, stopping about a half inch from the end of the bottom slide. Then the top slide is held loosely and dragged back with an uneven motion, so that a very thick layer of blood results.

The slides are dried as quickly as possible under an electric fan. They are then immersed in a solution of 1 per cent acetic acid in distilled water contained in a Coplin staining jar and allowed to remain for several minutes until dissolution of the red blood cells is effected; the addition of 5 per cent commercial formalin to this solution contributes to safety but is probably unnecessary and has the disadvantage of causing additional shrinkage of the leucocytes. The slides are washed through at least three changes of distilled water to remove all traces of acid (excess of acid interferes with staining).

The preparations are stained with Bordet-Gengou's carboltoluidine blue, as suggested by Dr. Alice Evans. This stain is superior to Hasting's or Wright's stains because cytoplasmic structures, such as normal and "toxic" granules, do not take the stain and hence cannot be confused with engulfed bacteria. Bordet-Gengou's toluidine blue is prepared by dissolving 5 Gm. of toluidine blue in 100 c.c. of 95 per cent ethyl alcohol, 500 c.c. distilled water, and 500 c.c. 5 per cent phenol. It should be filtered after standing for one or two hours, and diluted with two parts of distilled water before being used for staining. The slides are stained for fifteen seconds, and washed with distilled water.

Preparations made in this manner are easily read and interpreted after experience, for the leucocytes and bacteria stand out as deeply stained structures against a faint, blue background. If care is taken to select isolated leucocytes for examination, avoiding clumps of cells and agglutinated organisms, the degree of phagocytosis can be determined speedily and accurately.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**SYPHILIS, Serologic Discrepancies in, Crawford, G. M., and Ray, L. F. J. A. M. A. 113: 1715, 1939.**

Of 2,862 new patients admitted to the syphilis clinic at the Massachusetts General Hospital in a two-year period, 335 late cases showed a positive Hinton and a negative Wassermann reaction. This group was studied exhaustively for additional evidence of syphilis. Nearly 50 per cent of these patients were found to exhibit a history or physical or laboratory evidence of the disease along with a repeatedly positive Hinton reaction. Another 25 per cent were diagnosed as syphilitic on the basis of a persistently positive Hinton reaction alone. In 12.5 per cent a vacillating serologic reaction and lack of other evidence created indecision as to the presence or absence of syphilis. In the remaining 14.3 per cent the one initial positive reaction, followed by repeated negative reactions, was held to be a technical error.

The average age of the entire group was 42.8 years. The known average duration of syphilis in the clinically active group was 21.5 years in acquired cases. The average age of congenital syphilitic patients with active disease was 25.3 years, the oldest of whom was 30.

False positive reports were computed to allow for the widest possible margin of error. All undecided cases and all technical errors were included. Comparing this number with all new specimens examined by the laboratory during the time of the study, a figure of 0.42 per cent of false positives was obtained. This corroborated clinically the high degree of specificity attained by the Hinton test in congresses on serologic tests.

**STREPTOCOCCUS VIRIDANS, Bacteriemia Following Extraction of Teeth, Palmer, N. D., and Kemple, M. J. A. M. A. 113: 1788, 1939.**

Seventeen per cent of a group of 82 patients who had not more than two teeth extracted had transient bacteriemia. In 13.4 per cent of these the organism was *Streptococcus viridans*. In four cases of subacute bacterial endocarditis the onset of septic symptoms dates from the time of dental manipulation.

Bacteriemia is present in an appreciable percentage of cases of severe oral sepsis independent of operation. Organisms "leak" into the circulation from such foci. Traumatization of the diseased alveoli laden with *Streptococcus viridans* causes dispersion of these organisms through the blood stream in a high percentage of cases. The percentage is roughly parallel to the severity of the infection in the gums and to the extent of the operative procedure. This may be purely mechanical dispersion rather than invasion. In persons with a normal vascular system and a normal defense mechanism, this form of bacteriemia is relatively unimportant. The circulation is usually cleared of the invaders within a few minutes. Hypersusceptibility of the tissues due to long-continued oral sepsis is speculated upon by Cecil. In persons who have pre-existing rheumatic valvular lesions or congenital defects in the heart, localization of the organism on such vulnerable areas during the transient bacteriemia, which so often follows dental operations, may herald the beginning of an engrafted bacterial endocarditis.

**MALARIA, Sulphanilamide (Prontosil) in the Treatment of, Niven, J. C. Inst. M. Research, Federated Malay States, Bull. 4, 1938.**

An investigation is described in which 80 persons with acute malaria treated with Prontosil are compared with 68 persons treated with quinine dihydrochloride.

It is found that prontosil is not so efficient as quinine in *P. falciparum* malaria.

Prontosil is still less effective in *P. vivax* and *P. malariae* malaria.

Prontosil is not an efficient gametocide in either *P. falciparum* or *P. vivax* malaria. Mosquitoes fed on "crescent" carriers after seven-day treatment with prontosil were found to be readily infected.

No toxic effects were noted.

It is concluded that, although prontosil has some lethal action on malaria parasites, especially *P. falciparum*, it has no place in the practical treatment of malaria owing to its low efficiency, possible toxicity, and relatively high cost.

#### GONORRHEA, Sulphanilamide and Its Derivatives in the Routine Treatment of, in the Tropics, Poynton, J. O. Inst. M. Research, Federated Malay States, Bull. 5, 1938.

An attempt is made to show that sulfanilamide therapy is a better and cheaper routine treatment for gonorrhea in the hospitals of Malaya than other forms of treatment at present employed.

In a series of 98 persons treated with sulfanilamide, 70 per cent were clinically cured, the majority within a fortnight.

A series of persons treated with uleron showed this drug to be far less effective than sulfanilamide.

No dangerous toxic effects were seen either with sulfanilamide or with uleron, and minor effects, such as giddiness or fever, disappeared immediately on suspending the treatment.

The results suggest that the Tamils tend to respond less favorably and are more liable to minor toxic effects than are the Chinese.

Total and differential leucocyte counts before and after treatment showed, in the majority of instances, that with sulfanilamide therapy these counts tend to return to normal as the result of a course of treatment.

In using sulfanilamide as a routine treatment for gonorrhea it is important that a definite schedule be followed and that certain precautions be observed. These are discussed.

In the course of completing these series of cases it became clear that sulfanilamide therapy is far more popular with Asiatic patients than previously employed forms of treatment. It is suggested that if it were introduced as a routine it would incite afflicted persons to present themselves at an earlier stage of the disease, reducing the incidence of serious complications, the activities of quack medicine vendors, and possibly, over a number of years, influencing the incidence of the disease by reducing the number of chronic infections at large.

It is suggested that sulfanilamide is unsuitable for self-administration and should not be available for open purchase.

Other sulfanilamide derivatives have been the subject of favorable reports, in particular M & B 693. At present the price of the latter militates against its routine employment in tropical hospitals for the treatment of gonorrhea.

This investigation was completed in the middle of 1938. Since that time many papers of importance on the subject have appeared, especially in connection with the value of M & B 693.

#### BONE MARROW: Limitations of Biopsy of Sternal Marrow, Kandel, E. V., and LeRoy, G. V. Arch. Int. Med. 64: 121, 1939.

It is the authors' impression and belief that extremely thorough clinical investigation of patients robs biopsy of sternal marrow of much of its value. In their hands it has more commonly been a link, and often a superfluous link, in the diagnostic chain than it has been the sole means of establishing a diagnosis. The knowledge that potent products for the treatment of pernicious anemia convert the megaloblastic marrow to erythroblastic marrow within usually, twenty-four hours, is valuable in clinical research. It permits more rapid assaying of new remedies and allows one to expose his patients to a minimum of risk during the testing period. With severely anemic patients there is an obvious advantage in not waiting seven or ten days for the expected response from experimental therapy.

The techniques for biopsy of marrow, the normal differential counts of material so obtained, and the limitations of the methods are discussed.

The authors' experience is reviewed, and 28 examples illustrating the uses, benefits, and disadvantages of biopsy of sternal marrow are presented.

**RABIES, Improved Methods in the Diagnosis of, Willett, J. C., and Sulkin, S. E.** J. Am. Vet. M. A. 95: 659, 1939.

The white mouse is the animal of choice in the laboratory diagnosis of rabies, Swiss mice being of no practical advantage.

Demonstration of Negri bodies in the test animal (white mouse) is the only dependable criterion for a positive diagnosis, since clinical manifestations are frequently too variable and indefinite to serve any expedient means of diagnosis other than the guinea pig method.

A simple procedure is described for demonstrating rabies virus in grossly decomposed animal brains.

Ether in final concentration of 10 per cent exerts a definite bactericidal effect upon contaminated dog brains after exposure for two hours at 4° C. This concentration has no effect upon the virulence of the rabies virus.

A simple technique is described for removing animal brains.

The following method is recommended for treating contaminated brains before intracerebral injection into animals. The central portion of the hippocampus major (cornu ammonis) is dissected out, and a small piece removed for microscopic examination and for glycerination. The remaining portion of the horn is emulsified in 10 c.c. of nutrient broth and allowed to stand for one hour at room temperature. The supernatant liquid (after larger clumps have settled) is pipetted into a stoppered 15 c.c. (0.5 ounce) centrifuge tube, and 10 per cent ether (about 1 c.c.) is added. The tube is then shaken thoroughly and stored in the refrigerator for two hours. After centrifugation at low speed the supernatant liquid below the ether layer is removed for animal inoculation.

The following instruments are required: (1) A heavy board (2 inches by 12 inches by 16 inches) with two 6 inch nails, located 2 inches from one end and about 4 inches apart; (2) one scalpel (Bard-Parker No. 4 handle, No. 20 blade); (3) one pair of rubber gloves; (4) one tablespoon; and (5) one metal adjustable hack saw (8 inch blade).

The animal is placed on the operating table with its head between the nails on the paper-covered heavy board. The surface of the head is then thoroughly moistened with 4 per cent cresol. With a heavy scalpel a sagittal incision is made midway between the eyes and ears, cutting through the skin, fascia, and muscles of the cranium. The brain may be exposed by sawing sagittally through the parietal bones in the region of the frontoparietal suture. The nerves on the under side of the cerebrum, tentorium, and spinal cord can be severed by forcing the tablespoon along the floor of the cranial cavity. The brain is then lifted out and transferred to a receptacle which is provided for this purpose.

**LEPTOSPIROSIS, Canine, in the United States, Meyer, K. F., Stewart-Anderson, B., and Eddie, B.** J. Am. Vet. M. A. 95: 710, 1939.

The following procedures are recommended:

*Silver Impregnation Method for the Demonstration of Leptospira:*

1. Fix blocks of tissues, not exceeding 1 cm. in diameter, in 10 per cent formalin in distilled water for several days or weeks, preferably three months.
2. Harden in absolute alcohol for twenty-four to forty-eight hours.
3. Pass through alcohol (70 per cent, 50 per cent, 30 per cent) to distilled water until the tissues are freed from alcohol.
4. Immerse in a 0.25 to 0.5 per cent silver nitrate solution in distilled water for eighteen to twenty-four hours at 37° C. in the dark.
5. Wash thoroughly in distilled water, changing the water several times.
6. Place in 0.5 to 1 per cent hydroquinone in 50 per cent alcohol for eighteen to twenty-four hours at room temperature.
7. Wash in 70 per cent alcohol; dehydrate in 95 per cent and absolute alcohol; clear in xylol; embed in paraffin.
8. Prepare very thin sections (3 to 4 microns). Remove paraffin and mount in balsam.

*Schüffner Modification of Verwoort's Medium:*

Use 1.5 liters tap water (free from chlorine; rain water may be used instead of distilled water) and 1.5 Gm. Witte peptone. Boil.

Add 6 c.c. phosphate mixture composed of 0.35 Gm. potassium phosphate (monobasic), 1.33 Gm. sodium phosphate (dibasic), and 100 c.c. distilled water.

Boil. Add 300 c.c. Ringer's solution composed of 0.8 Gm. sodium chloride, 0.02 Gm. calcium chloride, 0.02 Gm. potassium chloride, 0.02 Gm. sodium bicarbonate, and 100 c.c. distilled water.

Continue boiling. Add 150 c.c. Sørensen's buffer solution, pH 7.2. Boil until precipitation is complete (about thirty minutes). Cool in icebox overnight. Filter. Test pH, which should be 6.8 to 7.2. Bottle. Autoclave at 15 pounds for fifteen minutes. The medium may be kept in this state in capped bottles until needed. For use add 8 to 10 per cent sterile rabbit serum, tube in 2.5 to 3 c.c. amounts, and inactivate for thirty minutes in a 56° C. water bath.

Test for sterility. Transplant, using a 1 c.c. pipette.

*Leptospira Agglutination Test—Porcelain-Plate Method:*

*Antigen:* Use a 4- to 6-day-old culture of each strain. Use both live and formalin-killed antigen (3 drops commercial formalin per 10 c.c. culture). Incubate plates containing live antigen for two hours at 37° C., and examine each dilution by dark field for lysis. Incubate plates containing formalin-killed antigen for four hours at 37° C. Examine each dilution by dark field for agglutination or clumping.

*Test:* For each serum specimen place four test tubes in a rack. Use Verwoort-Schüffner buffer solution without serum as diluent.

Tube	1	2	3	4
Buffer	1.2 c.c.	0.9 c.c.	0.9 c.c.	0.9 c.c.
Serum*	0.3 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Dilution	1:5	1:50	1:500	1:5,000

Use two porcelain plates for each specimen, one for live antigen, one for formalinized killed antigen.

**CL. TETANI, Serological Identification of, MacLennan, J. D. Brit. J. Exper. Path. 20: 26, 1939.**

The H and O agglutinin reactions of *Cl. tetani* have been examined, and the findings of Gunnison (1937) confirmed.

The O antigens of *Cl. tetani* are apparently species-specific, and have not been found in any of nine other species of terminally-spored anaerobes.

By means of suitable O antisera a new serologic type of *Cl. tetani*—type X—has been identified.

**TYPHOID BACILLI, A Leucocidal Toxin Extracted From, Dennis, E. W., and Senekjian, H. Am. J. Hyg. 30: 103, 1939.**

A potent leucocidal factor has been demonstrated in trichloroacetic acid extracts of typhoid bacilli.

Leucocidal extracts of bacilli are analogous to leucocidal culture filtrates in selective lethal effect on the pseudo-eosinophilic granulocytes, and in thermostability.

Purification of the trichloroacetic acid extract results in increased potency of the toxic somatic antigen-complex, but in loss of leucocidal activity. The crude extract represents a mixture of these two factors.

Typhoid leucocidin is a component of the typhoid bacillus, and its presence in culture filtrate is probably the result of early autolytic processes.

The leucocidal substance in trichloroacetic acid extracts is not in an antigenic state. Leucocidin can be demonstrated in similar extracts of *Salmonella typhimurium*, *Sal. schottmülleri*, *Sal. suispestifer* (voldagsen strain), *Sal. enteritidis*, *Sal. gallinarum*, and *Sal. paratyphi*, listed in order of decreasing leucocidal activity.

\*Transfer 0.1 c.c. from tube 2 to 3 and the same amount from 3 to 4.

**TUBERCULOSIS, Sulfanilamide in Clinical,** Nayer, H. R., and Steinbach, M. M. *Am. Rev. Tuberc.* 40: 470, 1939.

Eight patients with extensive bilateral pulmonary tuberculosis were treated with sulfanilamide for a period of from ten to seventy-one days.

As far as the authors could evaluate the results, there was no apparent effect, either beneficial or harmful, upon the disease.

**STREPTOCOCCI, Hemolytic, Studies on, From Human Sources,** Rudd, G. V., White, C., and Ward, H. K. *Australian J. Exper. Biol. & M. Sc.* 17: 25, 1939.

Seven hundred and eighty-four strains of group A hemolytic streptococci isolated in Sydney, Australia, were examined for their serologic type. Five hundred and eighty-nine belonged to one of Griffith's types, 92 belonged to types not represented in Griffith's classifications, and 103 could not be typed.

Griffith's types 2, 3, 13, 14, 15, 18, 19, 24, "Quinn," and "Coggins" have not been isolated in Sydney up to the present.

Types 17, 11, 1, and 4 accounted for 75 per cent of the scarlet fever strains, while these types accounted for only 12 per cent of strains from other sources.

Evidence is submitted suggesting that capsulated organisms are likely to be more infectious than noncapsulated organisms.

The technical problems of typing are discussed.

**BLOOD, Biochemical Changes Occurring During the Storage of Human,** Bick, M. *Australian J. Exper. Biol. & M. Sc.* 17: 321, 1939.

The storage of human blood is accompanied by a decrease in the glucose content of the blood, an increase in the reduced glutathione, uric acid, creatinine, nonprotein nitrogen, and inorganic phosphate. There is little increase in the urea content of the blood.

The onset of hemolysis may be delayed by the addition of glucose. The optimum amount of glucose is 0.6 to 0.7 per cent. A small part only of the increase in nonprotein nitrogen is accounted for by the increase in urea, creatinine, and uric acid. Hemolysis of the erythrocytes generally occurs when the nonprotein nitrogen reaches a concentration of 55 to 60 mg. per 100 c.c. blood.

The biochemical changes that take place may be attributed to the erythrocytes, but the essential nature of the process is unknown.

As the inhibition of hemolysis does not seem possible as yet, it should be stressed that the rigorous cleansing of all glass and purification of reagents must be undertaken to procure consistent and reproducible results in this work.

**HEMOPHILIA, A Study of the Clotting Defect in: The Delayed Formation of Thrombin,** Brinkhous, K. M. *Am. J. M. Sc.* 198: 509, 1939.

A quantitative study of changes in the prothrombin titer of hemophilic blood shows that its prothrombin is converted very slowly into thrombin. This delayed prothrombin conversion can be corrected by adding less than 1 mg. of crude thromboplastin to 100 c.c. of hemophilic blood. Evidence indicates that the prothrombin and fibrinogen are normal in amount and in reactivity in this disease. There is no excess antithrombin. Emphasis is placed upon the formed elements of the blood and the sluggishness with which they liberate thromboplastin.



## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

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### A Textbook of Pathology for Nurses\*

THIS is a well-written and well-organized little book, the purpose of which is to present to the student nurse in simple language, a working concept of the mechanism of disease.

Based upon the author's course in the Mt. Sinai Hospital of New York, it fulfils its purpose satisfactorily and should meet a favorable reception from instructors in training schools for nurses.

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### Pathology†

THE subtitle of this book, "An Introduction to Medicine and Surgery," indicates the manner in which the authors have approached their subject. For, as is now rather generally appreciated, pathology is no longer regarded as synonymous with morbid anatomy, but appreciated as an essential phase in the study of disease in the living patient.

In the organization of the book the authors have accepted a compromise between the conventional separation into general and special pathology and that treating as one all conditions due to the same cause. The result is an effective text, not only for the student to whom it is primarily addressed, but also for the physician at large.

It is recommended as a good book.

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### Proctoscopic Examination‡

BECAUSE of the generous margins and large type, this small book is even smaller than it appears to be. While there is much that is good in the text, the book gives one the impression of being an expansion of a synopsis for lectures and, as a whole, it is a somewhat fragmentary and uneven exposition of the subject. The chapters on proctoscopic examinations are the best portion of the text. There will be some reluctance to accept neoplasms as common causes of diarrhea, and in comparison to the space given to other conditions of importance (for example, amoebic dysentery and amoebic colitis) that given to the discussion of malignancy seems disproportionate though not in itself exhaustive.

This book could well be taken as a synopsis from which a better and more comprehensive work could be written.

As it stands, one must reluctantly admit that it is not among the outstanding contributions in this field.

\*A Textbook of Pathology for Nurses. By Coleman B. Rabin, B.S., M.D., Lecturer in Pathology, Mt. Sinai Hospital School of Nursing; Associate Radiologist to the Mt. Sinai Hospital; and Thomas B. Davie, M.D., M.R.C.P., Professor of Pathology, University of Liverpool. Cloth, 321 pages, 374 illustrations, including 5 color plates, \$10.00. P. Blakiston's Son & Co., Philadelphia, Pa. \$1.75. W. B. Saunders Co., Philadelphia, Pa.

†Pathology. An Introduction to Medicine and Surgery. By J. Henry Dible, M.B., F.R.C.P., Professor of Pathology, University of London; and Thomas B. Davie, M.D., M.R.C.P., Professor of Pathology, University of Liverpool. Cloth, 321 pages, 374 illustrations, including 5 color plates, \$10.00. P. Blakiston's Son & Co., Philadelphia, Pa.

‡Proctoscopic Examination and Diagnosis and Treatment of Diarrheas. By M. H. Streicher, M.S., M.D., Assistant Professor of Medicine, University of Illinois, College of Medicine and Research and Educational Hospital, and Department of Surgery, Grant Hospital of Chicago. Cloth, 150 pages, 29 illustrations, \$3.00. Charles C. Thomas, Springfield, Ill.

## Medicolegal and Industrial Toxicology\*

THIS is a compact and well-planned discussion based upon practical experience. While not encyclopedic in scope, it is in many respects a *multum in parvo*, as indicated by the chapter headings: Poisons and Drugs, Criminal Investigations, Medicolegal Examinations of Miscellaneous Nature, Industrial Poisoning, and Occupational Diseases.

As a ready reference text, to be supplemented by larger and more comprehensive volumes, this book should be of interest and value to industrial physicians, coroners, pathologists, lawyers, insurance adjusters, and all who are concerned with the fields covered. A good book to have at hand.

## Blood Groups and Blood Transfusions†

THIS book needs no introduction, for it has been, since the appearance of its first edition, a standard and authoritative text on the subject.

Extensively revised, the text is one which may well be said to be invaluable to the physician, clinical pathologist, medicolegal expert, and laboratory worker, and may justly find a place on the reference shelf of the legal library.

The section on blood transfusions has been rewritten to include the latest developments and constitutes a complete survey of the subject.

Blood grouping is thoroughly discussed with emphasis on its medicolegal implications as is also the grouping of blood stains. Research workers will find the discussion of blood groups in animals interesting and valuable.

All in all it is an excellent book and a "must" item.

## The Neurogenic Bladder‡

THE purpose of this book is to give the reader a working knowledge of the value of cystometry in the differential diagnosis between neurogenic and non-neurogenic disease of the bladder.

It is a well-written monograph of practical importance and value, and can be recommended as an authoritative study.

## Laboratory Diagnosis§

IT IS not surprising that this book, by reason of its practical approach to the problems of the student and to the problems of the physician as well, should have reached its third edition.

Increased in size and thoroughly and comprehensively revised, it retains the plan of its predecessors. The first section (322 pages) describes the clinical applications of laboratory procedures and their clinical interpretation. The remainder of the book is devoted to a description of technique.

\*Medicolegal and Industrial Toxicology, Criminal Investigation, Occupational Diseases. By Henry J. Ellmann, Ph.D., Director, Physician's Laboratory Service of Toledo, Ohio; Lecturer in Bacteriology and Histology, Mary Manse College of Toledo. 324 pages, \$3.00. P. Blakiston's Son & Co., Philadelphia, Pa.

†Blood Groups and Blood Transfusions. By Alexander S. Wiener, A.B., M.D., Serologist and Bacteriologist in the Office of the Chief Medical Examiner of New York City. Cloth, ed. 2, 306 pages, 52 figures. Charles C. Thomas, Springfield, Ill.

‡The Neurogenic Bladder. By Frederic C. McLellan, M.S., M.D., Instructor in Surgery, University of Michigan Medical School, Ann Arbor, Michigan. Cloth, 206 pages, 1 plate, 8 figures and numerous charts. Charles C. Thomas, Springfield, Ill.

§Laboratory Diagnosis. By Edwin E. Osgood, M.A., M.D., Associate Professor of Medicine and Head of the Division of Experimental Medicine, University of Oregon Medical School. Washable fabric, ed. 3, 676 pages, 10 colored plates, 27 text figures, \$6.00. P. Blakiston's Son & Co., Philadelphia, Pa.

The book is so well known that it needs no extended description, but it can be said that this edition is as well organized, well written, and authoritative as its predecessors; it will undoubtedly maintain and enhance the popularity already acquired. A good book for the student, an excellent book for the clinician, and a valuable addition to the laboratory reference shelves.

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### What's Your Allergy?\*

THIS is a book for the intelligent layman. While the title suggests a possible humorous approach, the book is a serious presentation in reasonably simple language, primarily for the enlightenment of the allergic patient. Statements made in the book are authentic, not exaggerated.

The chapter dealing with the mechanism and theory of anaphylaxis is obviously the most difficult portion of a semipopular book for presentation in simple and at the same time adequately descriptive phraseology. The author's solution to this problem of a clear-cut explanation of a subject, which physicians and even research students do not clearly understand, is not too involved and should leave the reader with the feeling that he has a fair idea of what may be happening in an anaphylactic reaction.

The reviewer fears that the authors may be placing too much hope in the therapeutic value of histamine, but the logic of their attitude is reasonable.

The book will make interesting reading for allergists, physicians in general, and non-medical readers.

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\*What's Your Allergy? By Lawrence Farmer, M.D., Chief of Allergy Clinic, Lenox Hill Hospital, New York; and George J. Hexter. Cloth, 234 pages, \$2.00. Random House, New York, 1939.

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## CLINICAL AND EXPERIMENTAL

### THE METEOROTROPISM OF ECLAMPSIA

PAUL G. FUERSTNER, M.D., SAN FRANCISCO, CALIF. AND FREDERICK SARGENT\*  
CAMBRIDGE, MASS.

THE influence of meteorologic changes on the outbreak and course of human diseases has long been acknowledged and for decades medicine has sought to explain this correlation. In this respect, perhaps eclampsia has been most frequently investigated, but although a large number of European workers have written regarding its relationship to changes in the weather, it has received less attention in the United States.

Only since a common basis of explanation for meteorologic factors has been found and made relatively easy to understand have the studies regarding the meteorotropism of eclampsia and other diseases received a new impetus. A short description of modern meteorologic conceptions is necessary before going on to a discussion of eclampsia and its relation to weather changes. De Rudder in Germany has written a book on meteorobiology of the human being, giving the medical man a concise explanation of the new concepts. Petersen's monographs *The Patient and the Weather* present a more exhaustive treatment of the same subject.

The most important cause of weather change is the passage of the so-called discontinuity surface or front which separates air masses of opposite characteristics. The two general types of air masses operating in this system are the polar air masses originating over the polar and the subpolar regions and the tropical air masses originating over the subtropical zone. The main differences between these air mass types when of continental origin are as follows:

#### POLAR CONTINENTAL

#### TROPICAL MARINE

Temperature	Cold	Warm
Humidity	Dry	Humid
Permeability to sunlight	Relatively transparent	Relatively opaque
Permeability to short wave rays	Strong	Poor
Ion content	High	Low
Electrical potential (Spannung)	Low	High

\*Mr. Sargent is attached to the Meteorological Department of the Massachusetts Institute of Technology.

Received for publication, July 5, 1939.

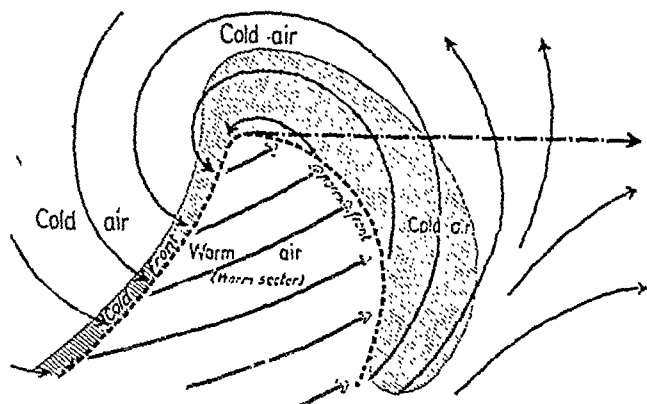


Fig. 1a.—The passage of warm and cold fronts as projected by Bjerknes.

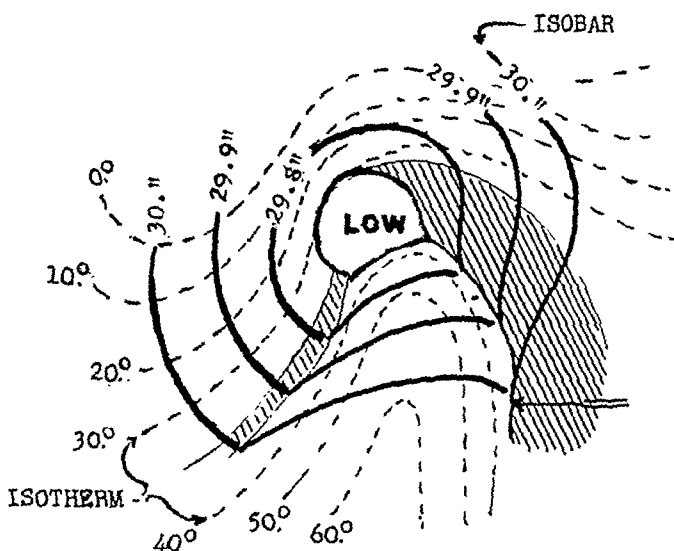


Fig. 1b.—The relationship of theoretical fronts to the isotherms and isobars.

Since these air masses mix to no appreciable degree, we generally find the discontinuity surfaces to be zones of rapid transition of the meteorologic elements. These regions of rapid transition are termed "fronts." The cold front is the discontinuity between the tropical current and the advancing wedge-shaped polar air mass. This wedge of cold air forces the warm air aloft, giving rise to clouds and precipitation. Often, too, the cold front is a rather turbulent affair, especially in the winter months. As we have indicated, the cold front is marked by a sharp drop in the temperature, a clearing of the air in a few hours and lowering of the humidity, and a steady rise in the barometric pressure. Generally the wind shifts from the southwesterly direction of the tropical current to the northwesterly direction of the polar current. The warm front, on the other hand, is the discontinuity surface between the receding modified cold air and the tropical current. The warm front is generally a more gradual transition than the cold front, and it is marked by increasing temperature and

humidity and a falling barometer. We shall refer to the period of weather from cold front to warm front as the "polar episode," and the period of weather from the warm front to the cold front as the "tropical episode." These two periods need not contain "pure" polar or tropical air masses, but they have been so labeled for convenience.

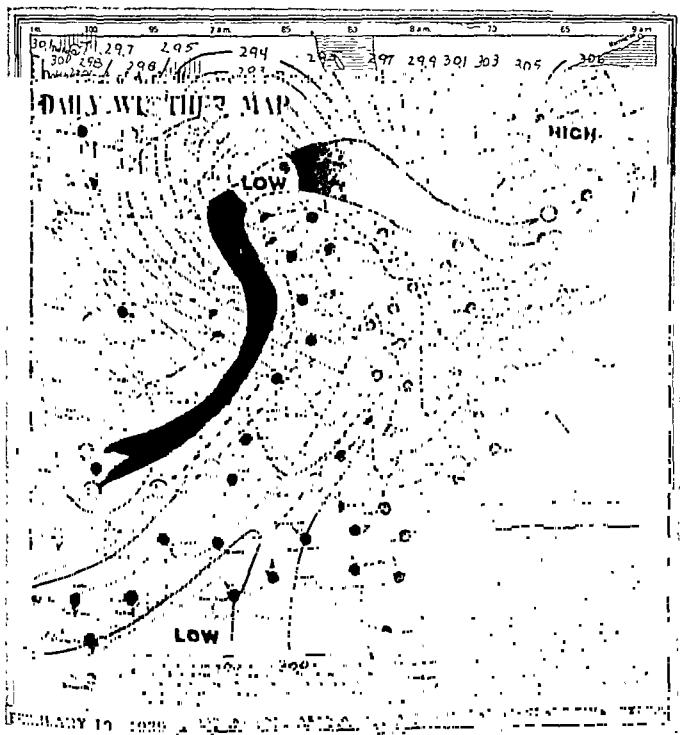


Fig 1c.—An actual frontal passage as reflected in a typical weather map. The episode is for February 10, 1939.

The frontal system is arranged in "depressions" or areas of low barometric pressure according to the idealized schemes outlined in Fig. 1, *a, b, c*.<sup>\*</sup> It must be emphasized that these diagrams are idealistic and that the daily analysis of the weather is by no means such a simple affair. This low pressure area moves generally from west to east, accompanied by most of our weather changes. There are, however, local weather changes that might also be concerned in the course of

\*The interested reader is referred to Horace Byers' *Synoptic and Aeronautical Meteorology*, New York, 1937, for a more detailed discussion of the modern point of view of air mass analysis.

disease, but in this paper we are interested only in the effect of weather changes—frontal passages—on the health of the maternal population.

The first studies concerning eclampsia and weather built upon the shortly characterized new meteorologic conceptions were carried out by Jacobs and von Heuss, who finally concluded that eclampsia follows only the infall of cold air. Von Latzka in Budapest came to the same conclusion. Eufinger and Weikersheimer, Ponzi, and others believe that the passing of warm fronts can have the same effect in producing convulsions, but in a considerably lower percentage. In a recent paper by Louros and Panajotou in Greece, the prevalence of the cold front influence, as described by Jacobs, is denied, and the "change of air masses" in either direction is emphasized as a cause for the outbreak of eclamptic convulsions.\*

Before going into our own studies on this question, it is necessary to consider one main difficulty concerning the reaction time elapsing between the meteorologic event and the onset of the eclampsia. When air mass changes follow each other at short intervals, which happens to be the case in Chicago where our material was collected, it is often not easy to decide if an eclamptic convulsion is associated with the passing of a warm front or with the invasion of cold air shortly preceding it. Jacobs, who acknowledges only a cold air invasion as the precipitating cause, still inculcates it even if there has been a definite passage of a warm front between the occurrence of the cold front and the onset of the convulsions. De Rudder states, with truth, that there is no reason why the warm front should not be acknowledged equally, because the main problem to be solved is whether *any* weather change takes place at the same, or approximately the same, time as the outbreak of the disease. A further difficulty consists in the fact that warm fronts are generally more gradual transition periods than cold fronts. The accurate determination of warm fronts, therefore, is considerably more difficult. Further, the fact may be stressed that the weather does not entirely consist of a series of abrupt air mass changes, but often the passage of fronts is so slow and diffuse that the real change of air mass is not so obvious.

Of course it would be ideal, as Dieckmann states in his recent paper, if one could collect the records of all toxemic pregnant patients for a city of several million people for comparison with the meteorologic data over the same period. Even then it would be extremely difficult to clarify the relations, because only the outbreak of convulsions is a clinical event definite enough to be reported with the exact time of its occurrence, and only these exact data are usable. (Since the prophylactic treatment of pregnancy toxemia has been steadily improving, convulsions occur more and more infrequently.) To deviate a moment: Exact data of this kind have been gathered in various other diseases which, like

\*The confusion may arise from the fact that with warm weather the patient becomes more alkaline, and under such conditions minor environmental alterations or disturbances may cause vascular spasm and initiate an eclamptic convulsion. This is possibly the reason for the earlier distribution of eclamptic deaths in the United States, to which Petersen called attention. Petersen assumes that vascular spasm is the underlying factor and has analyzed the mechanism as an autonomic dysintegration, most often precipitated by weather disturbance in susceptible individuals. (Petersen, William F.: *The Patient and the Weather*. Vol. II, Autonomic Dysintegration, Ann Arbor, Mich., 1934, Edwards Brothers; Vol. IV, Part 1, Carillo-Vascular-Retic Disease, 1936.)

eclampsia, are closely connected with the vascular system and are precipitated by a change of tonus controlled by the vegetative nervous system. This change in tonus, for instance in apoplexy, acute onset of glaucoma, acute laryngeal croup, spasmophilia, etc., has been definitely proved to follow a frontal passage.

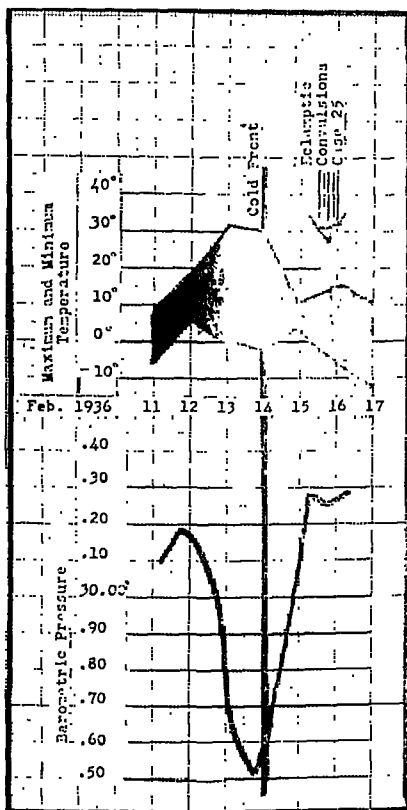


Fig. 2.

Out of a material of 425 cases of toxemia of pregnancy collected by Dr. F. H. Falls\* in the Cook County Hospital and the Research and Educational Hospital, University of Illinois, College of Medicine, Chicago, during the years 1931-1937 for other purposes, one of us (F.) chose 46 cases for this study. All these were eclampsia with convulsions. The high number of pre-eclamptic and toxemic patients could not be included for the simple reason that, as previously

\*Dr. Falls was kind enough to allow a study of his cases.



pointed out, the connection between weather and eclampsia primarily concerns the precipitation of convulsions. The clinical records of the 46 cases included the most exact data concerning the time of the outbreaks in hours and minutes. The corresponding weather charts were kindly provided by Dr. W. F. Petersen. Further, the official daily weather reports of the U. S. Weather Bureau were at our disposal.

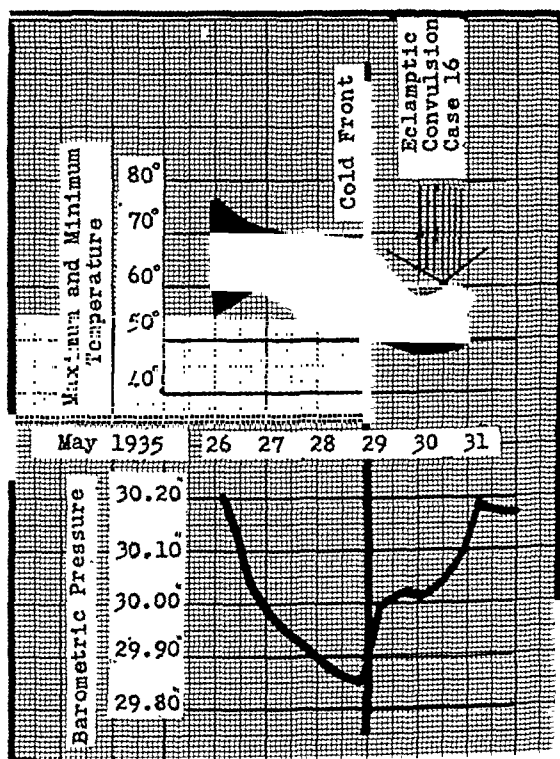


Fig. 3.

The 46 cases studied concerned 36 white and 10 colored patients. Thirty-one, or 67.4 per cent, were primiparas; 15, or 32.6 per cent, multiparas. Of the 36 white patients, 24, or 66.6 per cent, were primiparas; 12, or 33.4 per cent, multiparas; of the 10 colored, 7 or 70 per cent, were primiparas; 3, or 30 per cent, multiparas. The age distribution was as follows:

Below 20 years,	12 = 26 per cent
20 - 30 years,	17 = 37 per cent
30 - 40 years,	11 = 24 per cent
Over 40 years,	6 = 13 per cent

In the group between 30 and 40 were 2 primiparas. In 21, or 45.7 per cent, the eclamptic convulsions broke out ante partum; in 13, or 28.3 per cent, intra partum; and in 12, or 26 per cent, post partum. The percentage of post-partum eclampsia seems remarkably high.

Delivery was spontaneous in 15 cases or 42.9 per cent; labor was induced in 6, or 17.1 per cent; cesarean section was performed in 8, or 22.9 per cent; vaginal operations (forceps, version, and extraction) were carried out in 6, or 17.1 per cent. In 12 cases, as already mentioned, eclampsia broke out only after delivery.

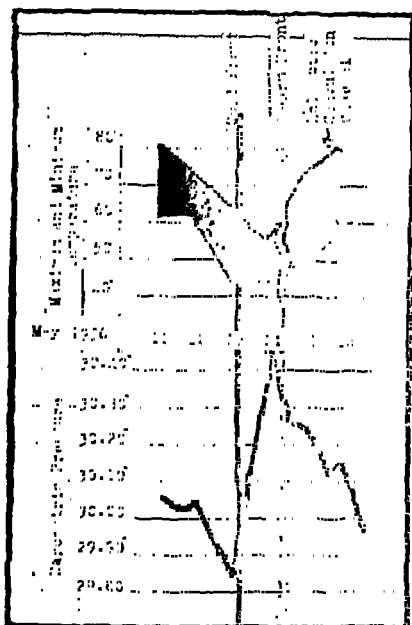


Fig. 4.

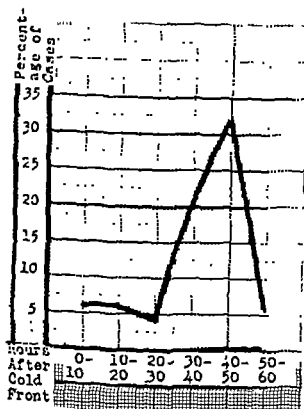


Fig. 5.

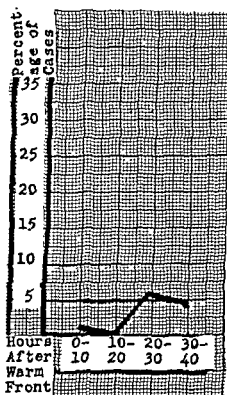


Fig. 6.

The fetal mortality was 10.8 per cent (5 cases), the maternal mortality 6.5 per cent (3 cases). The low percentage of fetal and maternal mortality speaks for the greatly improved treatment available. The number of observed convulsions ranged on an average between 1 and 9; in one case which ended well, no fewer than 54 convulsions occurred.

As far as the seasonal distribution is concerned, the highest number of cases was observed in April, as noted by Harrar and Davis in New York in 1926. A renewed increase in cases was found in August. In statistics concerning 36 cases of eclamptic death in 1932 in Chicago, the highest numbers were found in January, March, and October. Dieckmann reported different months of prevalence from the different regions of the earth. All these findings suggest that the season in itself is not associated with the disease, but the singular weather episodes which can be absolutely different in different years.

In ascertaining the association between the 46 eclampsia cases and changes of air masses, we obtained the following results: In 34, or about 74 per cent. of the convulsions were connected with infalls of cold air; in 6 cases, or 13 per cent, with passing of warm fronts. Figs. 2, 3, and 4 show how this was worked out. In the remaining 6 cases, or 13 per cent, the weather was too unsettled to permit definite conclusions. In 2 of these cases we are inclined to consider a polar front, and in 4, a warm front passage to be the factor. The following data refer to the interval between the meteorologic event and the outbreak of the convulsions:

0 - 10 hours after cold front:	3, or about 6 per cent
10 - 20 hours after cold front:	3, or about 6 per cent
20 - 30 hours after cold front:	2, or about 4 per cent
30 - 40 hours after cold front:	9, or about 20 per cent
40 - 50 hours after cold front:	14, or about 32 per cent
50 - 60 hours after cold front:	3, or about 6 per cent
Total during "polar episode"	34, or about 74 per cent

0 - 10 hours after warm front:	1, or about 2 per cent
10 - 20 hours after warm front:	0, or about 0 per cent
20 - 30 hours after warm front:	3, or about 6 per cent
30 - 40 hours after warm front:	2, or about 4 per cent
Total during "tropical episode"	6, or about 13 per cent

The curves in Figs. 5 and 6 illustrate these data. Thus the evidence shows that much the highest percentage of cases are distinctly correlated with the passing of a cold front and that the highest number of onsets occur within forty and fifty hours after the invasion of cold air.

As previously stated, our study of the association between weather changes and eclampsia has been confined to the precipitation of convulsions. We have no intention of claiming that the weather has any etiologic significance with regard to the disease itself. The etiology of eclampsia is, in spite of the existing large number of old and new theories, still an unsolved problem. The only fact beyond any doubt is that it consists in a severe metabolic disturbance of the maternal organism. As far as the mechanism of the precipitation of eclamptic convulsions is concerned, one can conclude that some factor influencing the vegetative nervous system of the toxemic or pre-eclamptic patient plays the main role. It is easily understood that sudden changes in the physical milieu produce these vegetative disturbances which can be counterbalanced by the normal

healthy organism, but not by the metabolically disturbed organism. Haselhorst, Hinselmann, Nevermann, and Heynemann proved that the lability of the capillaries is increased in eclamptic patients. Petersen states that weather changes precipitate even in the normal organism definite alteration in metabolism. The results of his extensive research in this matter show that cold air invasions precipitate contraction of the skin capillaries, and a rise in blood pressure; further, hyperglycemia, a relative alkalosis, and a fall in the carbon dioxide tension in the blood. He expresses the sum of all the physiologico-chemical changes precipitated by a cold front as the ARS phase ( $A = \text{anabolism}$ ,  $R = \text{reduction}$ ,  $S = \text{spasm}$ ). Opposite physiologico-chemical effects occur with the passage of a warm front. They are designated by Petersen as the COD phase ( $C = \text{catabolism}$ ,  $O = \text{oxidation}$ ,  $D = \text{dilatation}$ ).

It is possible that electrical changes which occur in the atmosphere during the passage of a front influence the labile organism of the toxemic pregnant patient. Elster and Geitel (quoted by de Rudder) have described the so-called "ions of the atmosphere." They consist of electrically loaded particles which are suspended in the air. Louros and Panajotou agree with Piéry in France who states that these ions have definite influence upon the lability of the vegetative nervous system. Düll and Düll have sought to associate solar eruptions, which cause electrical magnetic disturbances upon the earth. These solar eruptions are, in their opinion, responsible, not only for the atmospheric occurrences, but also for the reaction of the living organisms. At present we are only just entering this new field of research, but one can already foresee that highly interesting results will be obtained in the near future.

Even from the practical point of view the conclusions obtained are of some value. It is interesting that in a recently published pamphlet on *Weather and Season in Surgery*, by Maurer, a certain number of prophylactic and therapeutic measures are recommended in consideration of the association between weather and disease. As early as 1932, Kümmel recommended that major surgery, with the exception of urgent cases, should not be performed during frontal weather. Similarly, it should be possible to work out some practical prophylactic measures against the precipitation of eclamptic convulsions through weather changes. A well-organized service for "prenatal care" should pay attention to the weather conditions. Imminent front passages are known to the official Weather Bureaus early enough to be communicated to the different prenatal services. Known toxemic or pre-eclamptic patients could be especially examined at these danger periods and proper prophylactic treatment could be administered. In this way the number of cases with convulsions could be further diminished.

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## STUDIES IN CELLULAR EXUDATES OF BOWEL DISCHARGES\*

### I. CONTROL OBSERVATIONS IN 1,123 PATIENTS, 7 AUTOPSIES, AND 3 DOG EXPERIMENTS

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#### INTRODUCTION

THE diagnosis of pathologic conditions of the bowel can be simplified by an understanding of the significance of cellular exudates in bowel discharges. Early work establishing the differential diagnosis of amoebic and bacillary dysentery on a basis of the cellular exudates in the diarrheal discharges in those two conditions has led to the wider study of cellular exudates in bowel discharges in order to determine their importance as a diagnostic procedure.

The studies which are being reported in this series include control observations in so-called normal individuals, that is, in those who state that they have no bowel complaints of any sort, and autopsy findings in man and in the dog. In the near future the series will be supplemented by the presentation of observations, now in process of completion, on the various cytologic pictures that are to be found. The results will be given of cellular exudate studies in patients with various types of bowel complaint. It is hoped that this work will make it possible for pathologic change in the bowel to be diagnosed with a greater degree of accuracy than has been practicable hitherto.

#### HISTORICAL

The diagnostic significance of cellular exudates in bowel discharges has long been recognized. Dutcher<sup>1</sup> was a pioneer in the field, and his contributions to the subject made as far back as 1903 (referred to by Bahr<sup>2</sup>)

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were followed by the early work of Jurgens.<sup>3</sup> In 1910, during an epidemic of dysentery in Fiji, Bahr demonstrated the practical value of the microscopic examinations of the stools for cells. Renewed interest in the subject came in 1917 when Bartlett<sup>4</sup> made a report on the pathologic aspects of dysentery from which the British Mediterranean Expeditionary Forces were suffering. He regarded amoebic dysentery as the major factor in the depletion of the ranks of the soldiers. Bahr and Willmore<sup>5</sup> replied to Bartlett's report in detail and showed that many cells were of tissue origin and were not *Endamoeba histolytica*, as Bartlett had asserted. They acknowledged that these cells could be mistaken for amoebae very easily and this error was responsible for much confusion in differential diagnosis. Wenyon and O'Connor<sup>6</sup> independently investigated the same problem and reached the same conclusions as Bahr and Willmore, but intimated that they thought the cellular exudate studies of bowel discharges might be of value in the diagnosis of bowel conditions other than amoebic and bacillary dysentery. Subsequent writers, including Graham,<sup>7</sup> Willmore and Shearman,<sup>8</sup> Anderson,<sup>9</sup> and later, Callender,<sup>10</sup> Haughwout,<sup>11</sup> and others, have made outstanding contributions in confirmation of the earlier work done in the field. As a result of these investigations, it is now an accepted fact that there are definite cytologic pictures in amoebic and bacillary dysentery and that these can be differentiated. The success which has attended the work on dysentery has stimulated inquiry into the relationship of cellular exudates to ulcerative colitis and to other types of bowel complaints. The early suggestion of Wenyon and O'Connor has been amply confirmed in the investigations herewith reported.

The special studies on cellular exudates, which have been carried out during the past four years, have established the fact that whenever pathologic change takes place in the bowel mucosa, cells of various types will be found in the discharge. Conversely also, when no cells are found in the bowel discharge, no pathologic condition is present. The presence or absence of these cells can be determined readily by microscopic examination of the bowel discharges. While this method of examination is one of the simplest, it is also one of the most effective available for the diagnosis of pathologic conditions of the bowel.

#### CELLULAR EXUDATES AND THE NORMAL BOWEL

A series of observations was made to determine first of all whether or not cellular exudate would be found in the discharge of patients whose bowel functions were reported to be normal. In order to establish a reliable check upon these observations, a series of autopsy examinations was also made. The control observations were made on a group of 1,123 patients selected at random from the venereal disease clinic at the Meinhard Health Center of the Department of Health of the City of New York. These patients were studied as part of an extensive survey which is being carried out by the Parasitology Service of the Bureau of Laboratories, Department of Health, City of New York, John L. Rice, M. D., Commissioner. They were selected because on close questioning they stated that their bowel functions were absolutely normal and also because they affirmed that they had no complaints which could be referred to the gastrointestinal tract.

The specimens examined consisted of, first, the normally passed bowel evacuations and, second, the diarrheal discharge following dosage with Epsom salts. So that the freshly passed warm specimen could be studied, patients were asked to come to the laboratory to evacuate the bowels following the administration of the salts.

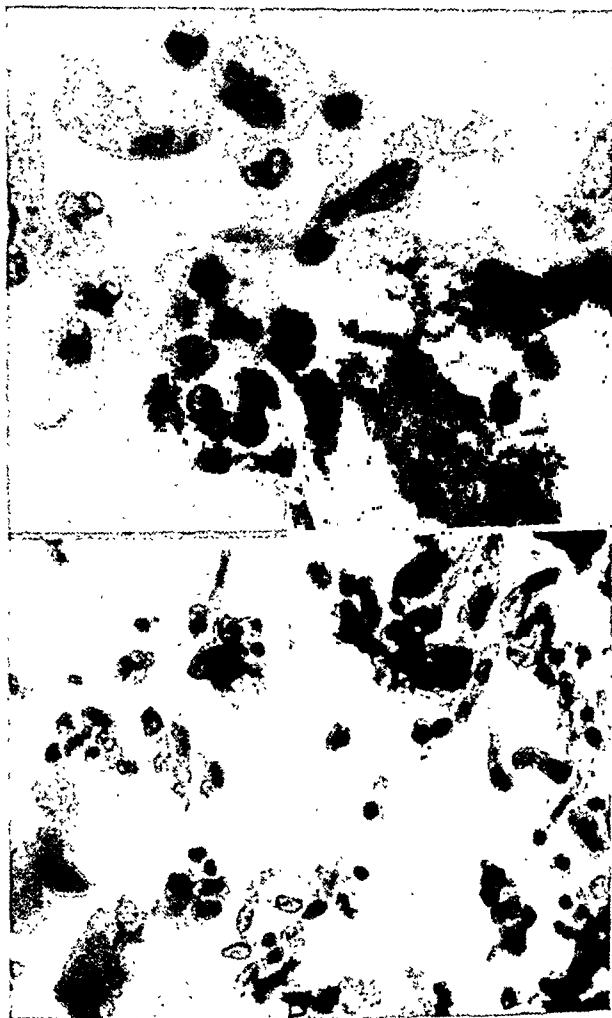


Fig. 1.—A, Degenerate cells found at autopsy in the bowel contents. Heidenhain's iron-hematoxylin stain. Oil immersion  $\times 970$ ; B, Cells found in scrapings from the bowel wall in the same patient at the same level as A. Heidenhain's iron-hematoxylin stain. High dry  $\times 470$ . Compare with Fig. 2 cellular exudates from patients with chronic ulcerative colitis.

Human autopsy examinations were made through the courtesy of Dr. Auerbach, Pathologist at Seaview Hospital, and Dr. Ward J. MacNeal, Director of the Department of Pathology, New York Post-Graduate Hospital. Autopsy specimens included bowel contents and scrapings from several locations along the gastrointestinal tract. In each case scrapings from the bowel wall were taken from the same place as the bowel contents, and were placed in suitably marked bottles which contained 10 per cent formalin. This procedure made it possible to examine the fecal matter and the bowel wall to discover whether or not cells were present, and, if present, to compare the cells

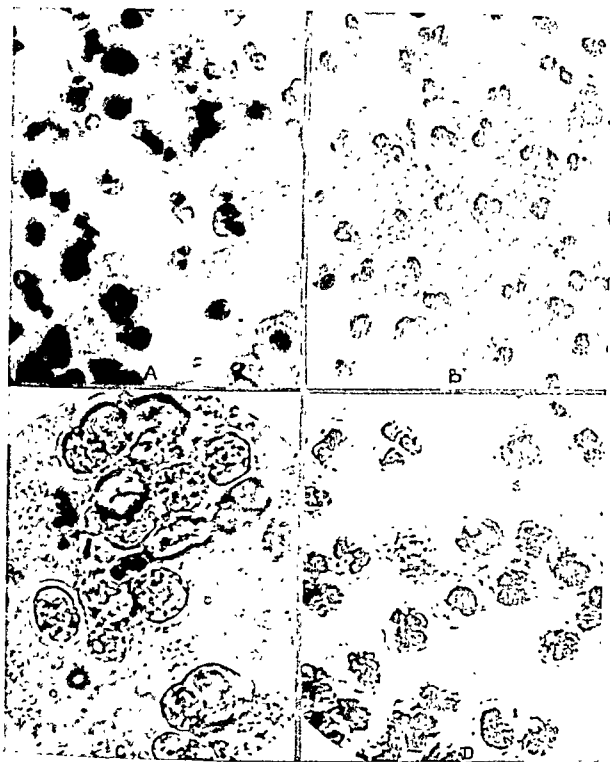


Fig 2.—Cellular exudates from cases of chronic ulcerative colitis. Oil immersion  $\times 970$  A and B Heidenhain's iron-hematoxylin stain. C and D methylene blue.

found in each. It was exceedingly difficult to secure autopsy material that was fresh and had not undergone an excessive amount of post-mortem degeneration and sloughing. However, for the purpose of this study, which was to determine whether or not cells normally occurred in fecal matter, an investigation was made to discover whether or not the cells found in the contents corresponded exactly to those found in the scrapings from the same area. If so, it was assumed that those cells were probably a result of post-mortem changes rather than an actual cellular exudation from the bowel wall during life.

In order to deal with the problems raised by post-mortem degeneration, three dogs were given lethal doses of sodium amytal intravenously. Their abdomens were opened immediately and their bowels were studied before any post-mortem changes could possibly take place. In each instance, the blood had not even coagulated but flowed freely from the congested mesenteric vessels.



Specimens of the bowel contents in various locations in the colon, as well as scrapings from the bowel wall at those locations, were taken for study.

#### TECHNIQUE OF EXAMINATION

A wet cover slip preparation made up with methylene blue was used to prepare the freshly passed bowel specimens for examination. This technique proved to be the most satisfactory after an extensive study was made of several thousand microscopic examinations of specimens which had been prepared with many different dyes and by various methods.

A small portion of the bowel discharge should be taken on a wooden applicator (a toothpick or match will do) or on a platinum loop, and should be mixed with Loeffler's methylene blue on a glass slide and then covered with a cover slip. The specimen should not be allowed to dry at any time; otherwise, the cells will degenerate into an unrecognizable mass. Using the low-power magnification, the entire cover slip is examined minutely in order to determine the amount of cellular exudate. In order to study the cytologic structure, individual cells are examined with the high dry and oil immersion lenses.

Permanent preparations can be made by smearing a portion of the discharge onto a clean glass slide. This should then be fixed wet in Schaudinn's solution, and stained with Heidenhain's iron-hematoxylin technique, similar to that used in the study of protozoa. The technique used in the cellular exudate study is as follows:

#### *Schaudinn's Solution*

- 2 parts of saturated mercuric chloride solution
- 1 part absolute alcohol
- 5 c.c. glacial acetic acid to 100 c.c. of the above mixture

NOTE.—Prepare fresh each day before use.

#### *Staining Method*

1. Schaudinn's solution heated to 60° C. for 15 to 20 minutes.
2. Rinse in 50 per cent alcohol, then immerse successively into:
  - 70 per cent alcohol iodine, for 2 minutes;
  - 70 per cent alcohol, for 2 minutes;
  - 50 per cent alcohol, for 2 minutes.
 Rinse in water.
3. Mordant 2 to 4 per cent iron alum at 30° C. for 10 minutes (short 2 minutes).
4. Wash well in running water for 5 minutes.
5. Stain in 0.5 per cent Heidenhain's hematoxylin at 30° C., for 30 minutes (short 2 minutes).
6. Wash well in running water for 5 minutes.
7. Decolorize in 2 to 4 per cent iron alum.

WATCH UNDER MICROSCOPE TILL NUCLEI ARE CLEARLY VISIBLE.

8. Wash well in running water for 5 minutes.
9. Immerse successively in 50 per cent alcohol for 2 minutes;
  - 70 per cent alcohol for 2 minutes;
  - 85 per cent alcohol for 2 minutes;
  - 95 per cent alcohol for 5 minutes;
  - absolute alcohol for 5 minutes;
  - absolute alcohol plus xylol for 5 minutes;
  - xylol for 5 minutes.
10. Heat Canada balsam and mount.

In many instances it was necessary to preserve specimens in 10 to 20 per cent formalin. When this was done there was no loss of cells through degeneration. Examination of specimens preserved in formalin was undertaken over a period of six months, and with the methylene blue wet cover slip preparation it was possible to observe the cellular exudate without difficulty.

#### RESULTS

Table I gives the results of the examination of 2,158 specimens obtained from 1,123 patients who stated that they had no complaints which could be referred to the gastrointestinal tract. Nine hundred ninety-six patients presented 1,960 specimens of normal bowel evacuations. Epsom salts were given to 127 patients who came to the laboratory and passed 198 specimens of watery stools.

TABLE I  
EXAMINATION OF SPECIMENS FROM PATIENTS WHO HAD NO COMPLAINTS REFERABLE TO THE GASTROINTESTINAL TRACT

	NORMAL BOWEL EVACUATIONS	DIARRHEAL BOWEL MOVEMENTS FOLLOWING EPSOM SALTS	TOTAL
Patients	996	127	1,123
Examinations positive for cells	7	4	11
Examinations negative for cells	1,953	194	2,147
Total examinations	1,960	198	2,158

Out of a total of 2,158 examinations, only 11 were positive for cells. The positive examinations were of specimens obtained from two patients, 57 and 62 years of age. Although an attempt was made to study these two patients further, it has been impossible to get in touch with them.

When 2,147 out of 2,158 specimens were negative for cells, the results would indicate that cells do not occur in the bowel discharges of normal individuals, even though Epsom salts are given in order to produce a diarrheal movement.

#### AUTOPSY EXAMINATIONS

With a view to testing the validity of the conclusions drawn from the studies with the so-called normal individuals, which showed that cells were not present in the bowel discharges of normal individuals, specimens of the bowel contents, as well as scrapings from the bowel wall at various locations, were taken during autopsies performed upon seven individuals. The purpose of this procedure was to discover whether the cells that appeared in the bowel contents were exactly similar to those of the scrapings so that the inference could be drawn, with a reasonable degree of confidence, that the presence of cells was due to post-mortem degeneration.

From this study it was discovered that the cells found in the bowel contents were exactly the same as the cells found in the scrapings taken from the same areas. Thus it can be affirmed that the cells were the result of post-mortem degeneration and that they were not present before the post-mortem degenerative processes began. These findings are in complete agreement with those of Schmidt and Strausburger,<sup>12</sup> given in their classic publication of 1910.

TABLE II  
AUTOPSY FINDINGS IN 7 PATIENTS

LOCATION	BOWEL CONTENTS			SCRAPINGS OF BOWEL WALL		
	TOTAL SPECIMENS	CONTENTS		TOTAL SPECIMENS	SCRAPINGS	
		POSITIVE	NEGATIVE		POSITIVE	NEGATIVE
Stomach	4	4	0	4	4	0
Duodenum	4	4	0	4	4	0
Jejunum	4	4	0	4	4	0
Terminal ileum	7	4	3	7	7	0
Cecum	6	4	2	6	6	0
Hepatic flexure	7	3	4	7	7	0
Transverse colon	6	4	2	5	5	0
Splenic flexure	6	4	2	6	6	0
Sigmoid	7	5	2	7	7	0

Moreover, the cells found in the scrapings from various levels of the bowel are entirely different from the cells found under pathologic conditions in the bowel discharges of living persons. The four chief types of cell found in the scrapings were (1) large wedge-shaped cells with granular cytoplasm and large oval nuclei composed of heavy rings and containing granules; (2) tall, columnar cells with a finely granular cytoplasm, the nuclei of which were large and oval-shaped with heavy rings and containing granules; (3) cells, irregular in shape, with a granular cytoplasm; their nuclei were oval to round but had very delicate rings which constituted the nuclear membrane; granules were included within these nuclei also; and (4) square cells with granular cytoplasm and small, round, solid nuclei. Polymorphonuclear leucocytes, small round cells, or macrophage cells were not at any time observed in the scrapings. The findings were the same for specimens in the wet methylene blue cover slip preparation as for those which were stained with iron hematoxylin in order to obtain permanent mounts.

#### DOG AUTOPSIES

Because patients had died of pathologic conditions that might have rendered the bowel tract abnormal, and also because in each instance several hours had elapsed between the time of death and the performance of the autopsy examination, the comparative study of bowel contents and scrapings was necessary. But in order to clarify the problem still further, three apparently normal dogs were given lethal doses of sodium amytal intravenously. As soon as respiration had ceased and before the blood had begun to coagulate, their abdomens were opened. Specimens of bowel contents and scrapings of the mucosa were taken from three levels of the colon and also from the terminal ileum. In each instance the specimens of bowel contents were entirely negative for cells, while scrapings of the mucosa showed cells characteristic of each of the levels of the colon and the terminal ileum from which the specimens were taken.

#### SUMMARY AND CONCLUSIONS

Problems connected with the diagnosis of pathologic change in the bowel mucosa would be simplified considerably if accurate information were obtained by microscopic examination of the bowel discharges. In order to determine whether or not cellular exudates should be expected in normal bowel discharges,

control observations were made in 1,123 patients who stated that they had no complaints referable to the gastrointestinal tract. A total of 2,158 specimens were examined from these so-called normal patients and, with the exception of 11, they were negative for cells. The eleven positive specimens came from two patients, aged 57 and 62 years, who could not be traced for subsequent study. One hundred ninety-eight specimens were diarrheal movements following dosage with Epsom salts.

Bowel contents from several different levels in the gastrointestinal tract were taken during the autopsy examinations on seven individuals. Scrapings were obtained from the bowel walls at the same locations. The cells found in the bowel contents were exactly the same as those of the scrapings from the same location, while there was also evidence to prove that the cells in the bowel contents were the results of post-mortem degeneration.

Three dogs were given lethal doses of sodium amytal and their abdomens were opened as soon as respiration had ceased. Specimens of the bowel contents from three locations in the colon and from the terminal ileum were entirely negative for cells.

From the three sets of observations, the conclusion may be drawn that if no pathologic condition is present, cells will not be found when microscopic examination is made of the bowel discharges.

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## ELECTROCARDIOGRAPHIC CHANGES INDUCED BY EXERCISE IN THE DIAGNOSIS OF CORONARY INSUFFICIENCY

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THAT spontaneous attacks of angina pectoris will occasionally produce changes in the electrocardiogram resembling coronary thrombosis was shown by Feil and Siegel<sup>1, 2</sup> and Parkinson and Bedford.<sup>3</sup> The mechanism of such changes is believed to be the same in both cases, namely, local asphyxia and anoxemia of the heart muscle due to acute interference with the blood supply in a localized area of the heart, temporary in the first case and permanent in the second.

If such is the case, any method employed to bring about rapid local asphyxia or anoxemia of the heart muscle should produce electrocardiographic changes. Under equal adverse conditions local asphyxia or anoxemia should develop much faster and to a greater degree in the presence of coronary insufficiency than in the normal state. Electrocardiographic changes, therefore, developing under such conditions, should indicate the presence of coronary insufficiency.

The methods employed to bring about the acute local asphyxia and anoxemia of the heart were continuous rebreathing of the same air, used by Rothchild and Kissin,<sup>4</sup> and by Katz, Hamburger, and Schultz<sup>5</sup>; the use of epinephrine by Levine, Earnstein, and Jacobson,<sup>6</sup> and Katz, Hamburger and Lev<sup>7</sup>; breathing of air consisting of 10 per cent oxygen and 90 per cent nitrogen by Levy, Bruenn, and Russell<sup>8</sup>; and the exercise test by Wood, Wolferth, and Livezey,<sup>9</sup> Whitten and Herndon<sup>10</sup> and, to a limited extent, by Bierring, Larsen, and Nielsen.<sup>11</sup>

Of these methods, the first two were found to be unreliable and dangerous by Katz, Hamburger, and Schultz,<sup>5</sup> and by Katz, Hamburger, and Lev.<sup>7</sup> The sponsors for the exercise test claim to have observed definite electrocardiographic changes after exercise in some patients with angina pectoris which were not present in normal persons. Thus Wood, Wolferth, and Livezey found that normal individuals showed no important changes in the QRS complex. There was merely an occasional deviation of the electrical axis to the right. The T wave also showed no change from a positive to a negative phase. A negative T wave changed occasionally to positive or a positive wave became more prominent. In half of their anginal patients, on the other hand, they noted a depression in the S-T segment and changes in the T wave from positive to negative. Whitten<sup>12</sup> also found frequent changes in the T wave in the anginal cases after exercise, but added that "further observations will probably reveal that one or more of these changes produced in the angina cases by exercise may occur in other than anginal cases." Bierring, Larsen, and Nielsen observed electrocardiographic changes after exercise in four anginal persons and stated that a few controls did not show any changes.

The object of the investigation covered in this paper was to determine if exercise actually produced different electrocardiographic changes in an abnormal rather than a normal heart. The test was carried out on 112 persons. Fifty-four had normal hearts; 26 had hypertension, 9 had questionable coronary changes, and 23 had unmistakable evidence of coronary artery disease.

In each case a tracing was obtained in the three conventional leads just before exercise, soon after exercise, two minutes later, and in some cases, again in four to six minutes. Because electrocardiographic changes may occur on mere change in posture, as shown in a previous communication,<sup>13</sup> all tracings both before and after exercise, were obtained in the standing position.

In the noncardiac group there were cases of psychoneurosis, bronchial asthma, obesity, diabetes, and the menopausal syndrome. Thirty-five persons, 10 of whom were hospital interns, were perfectly normal individuals with no complaints. Of the 23 persons with definite evidence of coronary disease, 10 had effort angina, 1 had recurring attacks of typical angina occurring spontaneously, and the rest had such symptoms as effort dyspnea, weakness, gastric disturbances, or precordial discomfort coming on after exertion. In the hypertensive group, the usual symptoms were palpitation, weakness, occasional dyspnea, and precordial pain. Some persons were symptom free.

It will be observed that in our study we took persons with coronary disease as a group, and did not limit it merely to those patients with the anginal syndrome as previous authors have done. The reason for this is that we were interested in learning whether coronary insufficiency as such, regardless of what its clinical manifestations might be, will produce different electrocardiographic changes following exercise than otherwise normal hearts. This applies also to the hypertensive group where at least relative coronary insufficiency is assumed to exist in many cases.

#### ANALYSIS OF THE ELECTROCARDIOGRAMS

*P-Wave Changes.*—Only 27 persons showed changes in the P wave soon after exercise. Of these, 19 were normal, 5 coronary, and 3 hypertensive. The changes consisted of increase in its height from 0.5 to 1 mm. and change from a rounded to a more pointed form. In some the changes were still present at the end of four to six minutes. In others the voltage became lower than that of the pre-exercise level, by that time.

*Changes in the QRS complex* were observed frequently and consisted of diminution or increase in voltage and slight shift in the axis to the right or left. The incidence of changes in voltage is shown in Table I. Such changes occurred more often in the normal than in the coronary, questionable coronary, and hypertensive groups. No prediction could be made as to the type of change in the various groups.

Immediately after exercise more persons showed diminished rather than increased voltage. The reverse was true two to six minutes later. The maximum variation in voltage of the QRS complex in the lead showing the greatest change was 6 mm., the usual being 2 to 3 mm. The greatest fluctuations occurred in the normal group.

TABLE I

Number and percentage of persons in each group, showing no change, diminution, or increase in heights of the R and T waves in the three leads at various periods after exercise. The findings before exercise were used for comparison. All persons had records soon after exercise, but no records were obtained of some persons two to six minutes after exercise.

NO. OF CASES	DIAG- NOSIS	ELECTRO- GRAM OBTAINED	R I						R II						R III					
			NO CHANGE		DIMINISHED		INCREASED		NO CHANGE		DIMINISHED		INCREASED		NO CHANGE		DIMINISHED		INCREASED	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
54	Normal heart	Soon after exercise	8	14.9	40	74.0	6	11.1	14	26.1	31	57.3	9	16.6	10	18.5	23	42.6	21	38.9
		Two to six minutes later	19	35.3	13	24.0	22	40.7	17	34.2	12	20.3	25	45.5	14	26.0	18	33.3	22	40.7
26	Hyper- tension	Soon after exercise	4	15.3	20	77.0	2	7.7	9	34.7	13	50.0	4	15.3	8	30.7	10	38.6	8	30.7
		Two to six minutes later	10	38.6	6	22.8	10	38.6	11	42.3	6	23.0	9	34.7	13	50.1	8	30.7	5	19.2
23	Coronary sclero- sis	Soon after exercise	10	43.4	12	52.3	1	4.3	8	34.9	10	43.4	5	21.7	10	43.4	7	30.5	6	26.1
		Two to six minutes later	9	39.0	5	22.0	9	39.0	10	43.4	7	30.5	6	26.1	9	39.0	8	34.9	6	26.1
9	Question- able coro- nary	Soon after exercise	1	11.2	8	88.8	0		3	33.3	5	55.5	1	11.2	5	55.5	3	33.3	1	11.2
		Two to six minutes later	5	55.4	2	22.3	2	22.3	4	44.4	2	22.3	3	33.3	5	55.4	2	22.3	2	22.3

NO. OF CASES	DIAGNOSIS	ELECTRO-CARDIO-GRAM OBTAINED	T I						T II						T III					
			NO CHANGE		DIMINISHED		INCREASED		NO CHANGE		DIMINISHED		INCREASED		NO CHANGE		DIMINISHED		INCREASED	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
54	Normal heart	Soon after exercise	14	22.3	20	37.0	22	40.7	10	19.5	23	42.6	21	38.9	12	22.2	21	38.9	21	38.9
		Two to six minutes later	28	55.7	13	24.0	11	20.3	26	48.1	14	25.9	14	25.9	17	31.5	19	35.2	18	33.3
26	Hypertension	Soon after exercise	8	30.7	7	27.0	11	42.3	5	19.2	5	19.2	16	61.6	7	27.0	6	23.0	13	50.0
		Two to six minutes later	13	50.0	5	19.3	8	30.7	9	34.7	6	23.0	11	42.3	13	50.0	7	27.0	6	23.0
23	Coronary sclerosis	Soon after exercise	4	17.3	8	34.9	11	49.8	4	17.0	5	21.7	14	60.4	9	39.0	6	26.1	8	34.9
		Two to six minutes later	9	39.0	7	30.5	7	30.5	12	52.2	7	30.5	4	17.1	12	51.9	6	26.1	5	22.0
9	Questionable coronary	Soon after exercise	2	22.3	4	44.4	3	33.3	4	44.4	2	22.3	3	33.3	3	33.3	4	44.4	2	22.3
		Two to six minutes later	3	33.3	5	55.5	1	11.2	3	33.3	4	44.4	2	22.3	3	33.3	6	66.7	0	



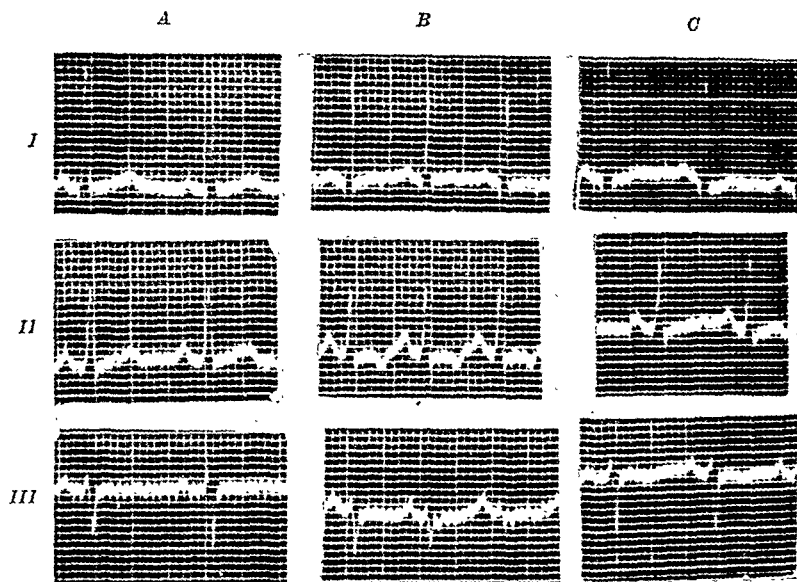


Fig. 1.—From a male 33 years old, with questionable coronary sclerosis. A, before, B, soon after, and C, three minutes after exercise. T-wave changes are noted in the first and second leads soon after exercise which did not return to the pre-exercise level in three minutes.

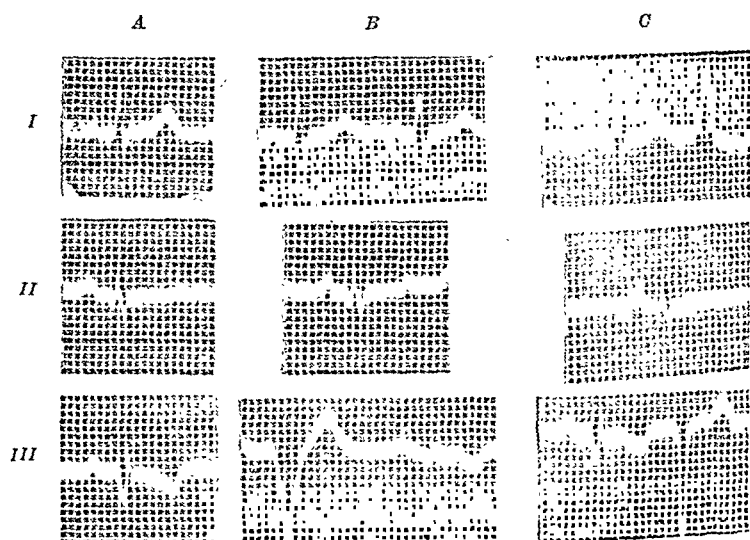


Fig. 2.—From a male 54 years old, with definite coronary disease and an attack of coronary occlusion three months before. A, before, B, soon after, and C, four minutes after exercise only trivial changes are noted in the T wave. QRS voltage in the third lead diminished and ventricular premature contraction appeared after exercise.

*Changes in the R-T or S-T segment occurred in 26 persons. The changes consisted of rounding, elevation, or depression. Of these, 6 normal persons, 3 with coronary disease, and 2 with hypertension had normal segments in one, two, or all three leads before exercise which became depressed or elevated soon after exercise, and in some cases remained so for two to six minutes. The greatest elevation or depression in any case was never more than 1 mm. Two normal persons had very slight depression and rounding before exercise, which returned to the isoelectric level afterward. One normal person had some depres-*

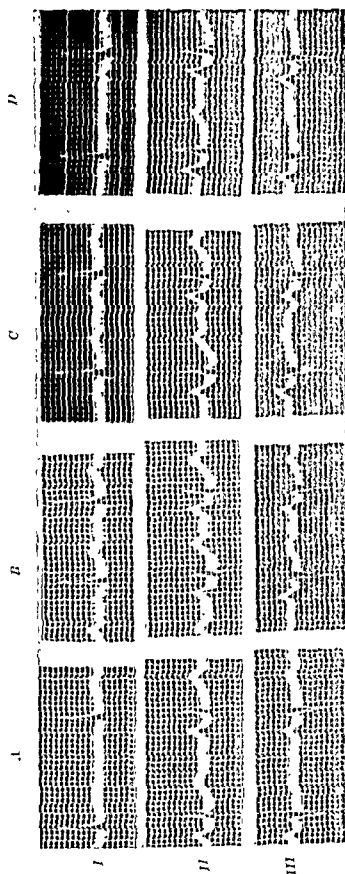


FIG. 3.—From a male 60 years old, with coronary sclerosis. A, before, B, soon after, C, two minutes after, and D, six minutes after exercise. The T wave is increased in voltage and is more pointed soon after exercise, remaining so two minutes after exercise. The electrical systole is shortened.

sion and another some elevation before exercise, which returned to the isoelectric level later. In one person with coronary disease, a pre-existing depression of the R-T segment in the second lead became more depressed soon after exercise and returned to the original condition two to six minutes later. There were 5 persons with coronary disease with slight depression of the R-T segment, one with elevation and 2 with rounding before exercise, which remained the same afterward.

Changes in the T wave occurred frequently and consisted of increase or decrease in voltage, more rapid conduction time, and slight change in appear-

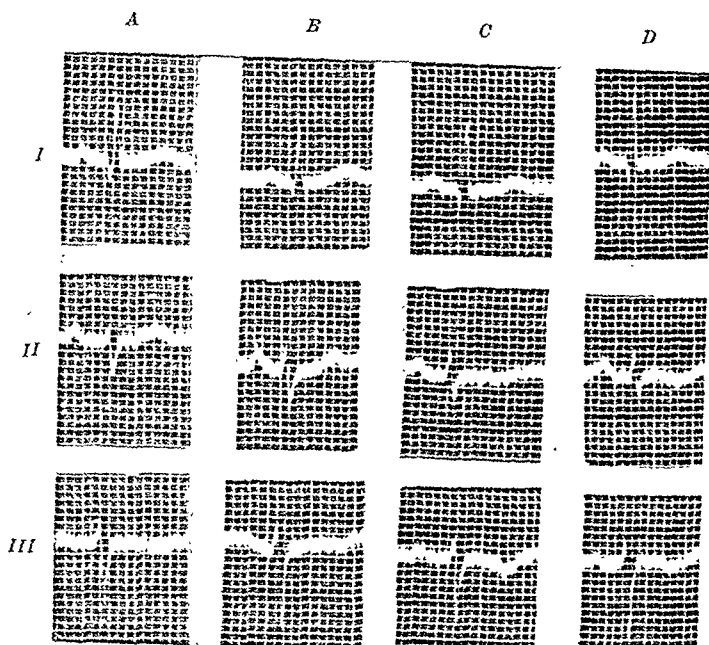


Fig. 4.—From a female, 48 years old, with moderate hypertension. A, before, B, soon after, C, two minutes after, and D, five minutes after exercise. There are changes from positive to negative T wave in second lead, and from mildly negative to greater negative T wave in third lead two minutes after exercise, remaining the same five minutes later. Slight change in QRS voltage is also noted.

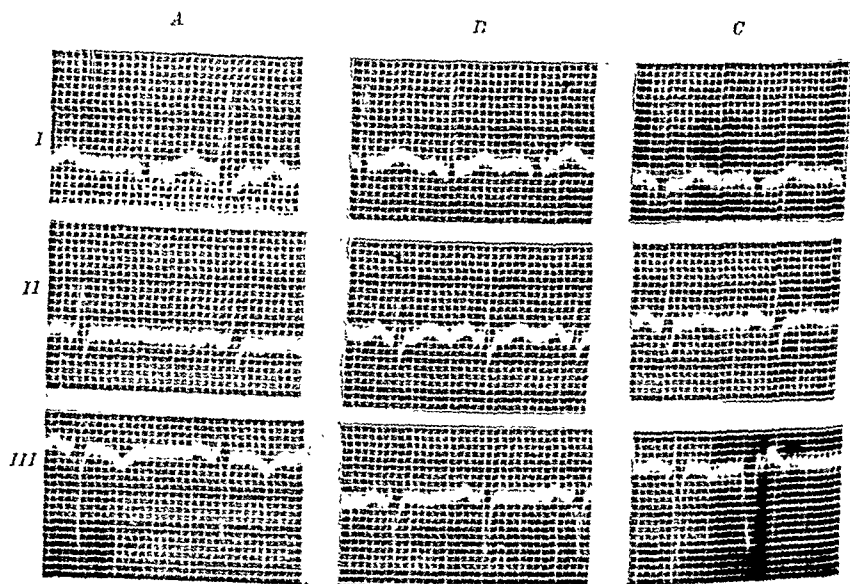


Fig. 5.—From a male 49 years old, with hypertension. A, before, B, soon after, C, two minutes after exercise. Voltage of QRS complex diminished and of T wave increased soon after exercise. The QRS returned to pre-exercise level in two minutes, but the T wave still remained somewhat higher at that time. A ventricular premature contraction is seen before and two minutes after exercise.

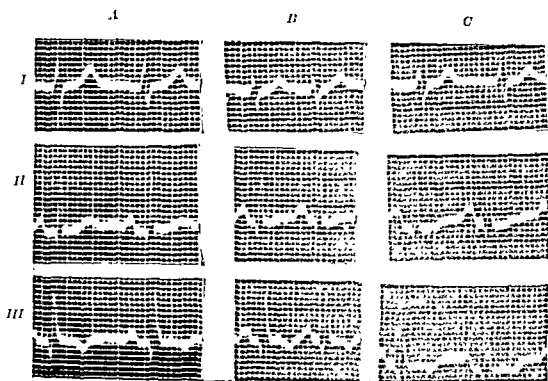


Fig. 6.—From a male 36 years old, with no clinical evidence of heart disease. A, before, B, soon after, C, two minutes after exercise. Voltage of QRS complex and T waves is diminished and there is some rotation of the axis to the right soon after exercise, remaining approximately the same two minutes after exercise.

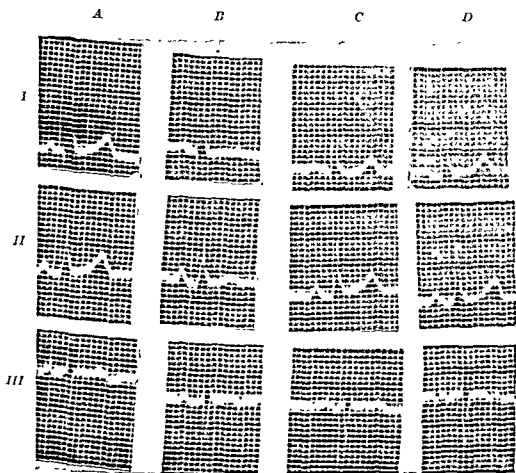


Fig. 7.—From a male 64 years old, presenting evidence of coronary and aortic sclerosis with the anginal syndrome. A, before, B, soon after, C, two minutes after, D, five minutes after exercise. Voltage of QRS and T is diminished soon after exercise and returns to pre-exercise level in two minutes, at which time the S wave in the third lead still remains of low voltage. In five minutes complexes are exactly the same as those in A.

ance from a rounded to a pointed form. As shown in Table I, the changes were observed more often in the first and second leads in the coronary group and in the third lead in the normal group. The maximum change in voltage was 1.5 mm., the usual being less than 1 mm.

In the normal group the T wave in the first lead never showed any changes from positive to isoelectric or negative. One person, however, showed such change in the second lead (Fig. 6) and six persons in the third lead. In six other persons an isoelectric T wave in the third lead changed to positive, and in nine persons negative T wave changed to isoelectric soon after exercise but returned to the original condition two to six minutes later. In five persons a positive T wave in the third lead before exercise remained so soon after, but was changed to negative or diphasic two to six minutes later.

There was one male, 33 years old, who complained of left precordial pain. Because his first heart sound was diminished in intensity and there was some peripheral arteriosclerosis, he was put in the group of questionable coronary disease. His electrocardiogram before exercise (Fig. 1) showed merely left axis deviation. Soon after exercise the T wave became almost isoelectric in the first lead and negative in the second lead, remaining so at the end of three minutes.

TABLE II

Persons in the various groups, showing range of heart rate, percentage increase in rate after exercise, percentage of persons returning in the pre-exercise level, and maximum percentage increase still present among those who did not return to pre-exercise level. The heart rates were calculated in all persons from the electrocardiograms obtained in the standing posture.

GROUPS	ORIGINAL HEART RATE	PERCENTAGE IN- CREASE IN RATE SOON AFTER EXERCISE	PERCENTAGE OF CASES THAT RETURNED TO PRE-EXERCISE LEVEL AT END OF		MAXIMUM INCREASE IN RATE OVER PRE-EXER- CISE LEVEL STILL PRESENT AT END OF	
			2 min.	4 to 6 min.	2 min.	4 to 6 min.
Normal	60 to 115	8.8 to 51.2	27.7	63.7	27.0%	25.6%
Hypertension	65 to 120	2.1 to 46.3	30.7	55.7	39.1%	23.0%
Coronary	60 to 125	6.5 to 43.8	34.7	81.3	20.0%	20.0%
Questionable coronary	92 to 115	10.2 to 46.5	44.4	72.9	29.5%	23.0%

In the group with coronary disease, one person (Fig. 7) had positive T waves in the first and second leads, which became almost isoelectric immediately after exercise and returned to the original condition later. In one patient a positive T wave in the first lead before exercise changed to isoelectric soon after exercise and was still in that condition two minutes later. In one person a diphasic T wave in the first lead before exercise changed to mildly positive after exercise and to greater positivity in two minutes. Another person, showing an isoelectric T wave in the first lead before exercise, became positive soon after exercise, and returned to isoelectric again two minutes later. Similar changes were noted in the second lead alone in two persons, and in the second and third leads in two other persons. All others in this group showed about the same T-wave changes as the group with normal hearts.

In the hypertensive group an isoelectric T wave in the second lead changed to positive immediately after exercise (Fig. 5) in two persons and two minutes after exercise in two others. Similar changes were noted in two persons in the

third lead. In one person a positive T wave in the second lead changed to diphasic soon after exercise and again to positive two minutes after. In four persons, the T-wave changes in the third lead were the same as in normal cases.

*Effect of Exercise on the Heart Rate.*—The acceleration of the heart rate following exercise is shown in Table II. It will be seen that individuals with normal hearts have a tendency for a somewhat greater acceleration soon after exercise, and such acceleration lasted longer than in persons with abnormal hearts. Inasmuch as the electrocardiograms from which the rates were determined were obtained in the standing postures, the rates in general are higher than the usual average.

#### DISCUSSION

If we consider the electrocardiographic manifestations occurring during the interval between the inception of the Q wave and the end of the T wave as the algebraic summation of spread and recession of the fractional components of the electrical potential in the individual cardiac muscle fibers in the ventricles, changes in the physical state of the heart muscle should theoretically produce changes in the resulting complexes.

Exercise is one condition where the physical state of the heart should undergo rapid change, and such change should occur earlier and be greater in coronary disease than in the normal heart. Anoxemia and the accumulation of waste products should occur much quicker and to a greater extent in coronary insufficiency. Theoretically, therefore, the exercise test should help us in the diagnosis of coronary insufficiency. Practically, however, no marked differences were noted in our findings in persons with normal and abnormal hearts following exercise. Comparatively few cases of coronary disease showed changes after exercise that were not found also in the normal.

That the heart rate, as determined from the electrocardiogram, did not return to the pre-exercise level in some cases as long as six minutes after exercise and that this occurred more frequently in normal hearts than in coronary disease appears to be contrary to belief that the heart rate must come down to the pre-exercise level within two minutes after exercise if we are to consider the heart normal. This might partly be due to the fact that all determinations were done in the standing postures.

In assigning significance to the findings presented in this paper, the amount of exercise employed in each case is probably an essential factor. Individuals who presented symptoms of dyspnea, palpitation, fatigue, precordial oppression or pain early did not do as much exercise as others. This may partly account for a more sustained effect of exercise on the electrocardiogram in the normal heart. If this be so, the subjective reaction of the individual, more than the electrocardiographic findings, should be considered the important criterion of myocardial insufficiency.

#### SUMMARY

Electrocardiographic studies were carried out in 112 persons before and after exercise. Fifty-four had normal hearts, 26 had hypertension, 23 coronary sclerosis, and 9 questionable coronary disease. Many of the persons showed increased voltage of the P wave, increased or diminished voltage of the QRS

complex, slight elevation or depression of the R-T or S-T segment, and increased or decreased voltage of the T wave. These changes occurred soon after exercise and lasted in some cases as long as six minutes. In two persons with coronary and in one with suspicious coronary disease a positive T wave in the first lead or first and second leads before exercise changed to negative or isoelectric T wave after exercise. In no normal persons did such changes occur in the first and second leads, but several showed it in the third lead. The usual changes were about the same in cases with normal as with abnormal hearts, except that in the normal heart they were somewhat more marked and lasted longer. In the normal heart there was a tendency for a greater increase in the heart rate after exercise.

We may conclude from these findings that the electrocardiogram does not usually show characteristic changes after exercise that could be taken to indicate the presence of coronary insufficiency. In an occasional case it may be of value. The subjective reaction is perhaps still the most important criterion of the cardiac functional state.

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# HYPERTENSION AND OBESITY\*

## A STATISTICAL AND CLINICAL STUDY OF 10,883 INDIVIDUALS

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TO WHAT extent is obesity related to hypertension? Medical literature does not conclusively answer this question. Although a wide variety of groups has been studied, there has been no standardization of either the hypertension or the obesity concept. The meaning of the term "obesity" is not clear. Surely weight alone does not measure it, and a clinical impression or a "build group," while helpful, is not a final definition of obesity. There is, therefore, need for a more adequate treatment of this subject.

Since the first rough studies of blood pressure were published, the most common associated physical finding has been overweight. Every study of the blood pressure-weight relationship has shown that the average blood pressure of a large group of persons rises with increments of weight. Yet there are conflicting opinions confronting the all-inclusive statement that blood pressure correlates to weight. Bowes<sup>1</sup> in 1917 found no weight relation to blood pressure. However, his group consisted of a small number of aged individuals, most of whom were afflicted with diseases other than hypertension alone. Post and Stieglitz<sup>2</sup> and Schulze and Schwab<sup>3</sup> reported groups of hypertensive persons in whom obesity was relatively rare. Geil and Secher,<sup>4</sup> though over 50 per cent of their hypertensive patients were obese, concluded that "ostensibly obesity has no significance in hypertension." Mosenthal,<sup>5</sup> while recognizing the relation, states that the influence of weight has probably been exaggerated.

The literature on obesity and hypertension is also confusing on the question of what ages are liable to the overweight-hypertension relationship. Symonds,<sup>6</sup> arranging his study by build groups on a weight per unit height basis somewhat similar to ponderal index, found an increase in pressure with an increase in relative obesity for all ages. The Actuarial Society of America,<sup>7</sup> on the other hand, found that two-thirds of all individuals ten pounds or more overweight have an elevated blood pressure, but specified that this relation existed only for ages over forty. Another confusion arises from the work of Huber,<sup>8</sup> who showed that 53 per cent of underweights and 6 per cent of overweights had low blood pressure, while 21 per cent of underweights and 14 per cent of overweights had high blood pressure.

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The source material for this study was gathered from records accumulated during the past ten years by the former Life Extension Institute, Chicago; the West Side Y. W. C. A., Chicago; the Student Health Service of the University of Chicago; and from the files of private practice.

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A survey of the literature on the problem can be broken into a number of subdivisions according to the methods of study. Some authors have used the clinical impression of obesity in its extreme state and have noticed, many times without specifically looking for it, the common association with an elevated blood pressure.<sup>9-13</sup> Conversely, other authors working with hypertensive persons have found a large percentage of their groups to be obese.<sup>14-18</sup>

Fewer data are available in regard to the opposite relationship between underweight and low blood pressure. The conclusions of Huber<sup>8</sup> and Brower<sup>19</sup> point out a very strong tendency for low blood pressure to be associated with underweight.

Another important source of data concerning the weight-blood pressure relationship has been furnished by the insurance studies. However, the insurance medical examiners have never used a standardized technique for recording weights. It has been only during the past few years that insurance examiners have carried scales. Yet even now scales are rarely used, and what is more, weights are never measured in the nude subject; hence a variable weight of clothing is recorded. In the past the insurance doctor merely asked the examinee what his weight was, and when in doubt, used the nearest drug-store scale. Medical directors themselves have recognized the unreliability of these weights, and, accordingly, have used a synthetic weight measure calculated from a formula embodying height, chest, and abdominal measures. It is obvious that such an indirect measure cannot give scientifically accurate results. Similar criticisms have been made on the lack of standardization of the technique of measuring blood pressures for insurance examination. The dissatisfaction of many medical directors with the standardization of their examinations is promising of conclusive work in the future. Bearing these criticisms in mind, the insurance studies have all shown that blood pressure increased with overweight and decreased with underweight.<sup>6, 20-24</sup>

A further criticism of much of the literature on weight and blood pressure is that there have been few studies that have established an index of obesity as differentiated from pure weight alone. Most of the work that has recognized the difference between weight and obesity has circumvented the problem by creating "build groups," accurate enough to differentiate gross extremes of obesity but not satisfactory in delineating the accumulation of adiposity within widely varying types of build.<sup>24-26</sup>

In order to overcome these criticisms and to determine more accurately the relationships inherent in the correlation of overweight and hypertension, we abstracted the periodic physical examinations of 10,883 individuals, apparently healthy or with minor complaints.\* In each case the examination was made under standardized conditions by one of six physicians. Identical methods were used in measuring the blood pressure, height, weight, chest, and abdomen, and an effort was made to control the conditions of examination as far as was possible. All physical measures were taken on the nude subject. Weight was measured on a beam scale made by the Continental Company, and recorded to the nearest pound for the purpose of this study. Height was measured on a specially designed apparatus wherein the heels of the subject were placed against the backboard of a platform and the shoulders thrown back against

\*For a complete analysis of the blood pressure distribution in these groups see Robinson, S. C., and Brucer, M.: The Range of Normal Blood Pressure, Arch. Int. Med., September, 1933.

a rod; the head was tilted to obtain the maximum height to one-fourth of an inch. The chest measure was taken with a steel tape. In the male it was taken about an inch above the nipple line during quiet breathing; in the female the chest measure was taken above the breast tissue well above the nipple line. The abdominal measure was at the level of the umbilicus. Blood pressure measurement was made with mercury manometers on the left arm of the sitting subject. The auscultatory technique and the fourth diastolic phase were used.

For the study of weight and blood pressure, 7,478 male and 3,405 female records were examined. The physical measures herein correlated were distributed in a manner to be discussed next.

#### PONDERAL INDEX AND BLOOD PRESSURE

Despite the obvious correlation of weight to blood pressure, conclusions drawn from the weight measure alone are highly inaccurate. Except at the extreme ends of an array, weight alone does not show a necessary correlation of obesity to blood pressure. For example, of two men—the one very short, the other very tall—each weighing 150 pounds, the short man would be overweight while the tall one would be underweight. This simple example makes obvious the necessity for rejecting weight as a single criterion of obesity. In order to relate obesity to hypertension it is first necessary to determine the degree of obesity within specific height groups. The clinical impression of obesity, as used by some authors, has palpable shortcomings, and likewise, the typing of individuals by build groups, as is practiced by insurance actuaries, is not entirely without fault, for this assignment of individuals into groups bears neither the statistical convenience nor the accuracy of a calculated index of relative obesity. Perhaps the simplest and most easily available method of ranging individuals relative to degree of obesity is with the use of the ponderal index. The ponderal index is defined as the simple ratio of weight in pounds divided by height in inches.\* Thus of two men, each weighing 150 pounds, the one 74 inches tall would have a ratio of 2.0, a lightweight index number, while the short one at 60 inches would have a ratio of 2.5, a heavyweight index number. It is easily seen why such an index is far more accurate and practical than weight alone, because it provides a single statistical unit combining height and weight, thus giving us a better numerical picture of obesity.†

\*Ponderal index has also been defined in terms of kilogram-centimeter ratio by some, chiefly European authors. The pound-inch unit, however, corresponds more closely to the American distribution of height and is in more common usage. The height distribution used in this study is very much like the distribution of height in the United States population. The mean height of the male sample was 68.1 inches. A standard deviation of 2.8 inches was shown. The mean female height was 65.6 inches. A standard deviation of 2.5, showing the female height to be somewhat shorter than the male.

†Because weight is tridimensional, it has been suggested that the cube of weight be used in calculating the index. Height factors may correct the small error present in the weight-height index. The coefficients of correlation of these various modifications of the weight-height index are as follows:

	Correlation	7,478 Men	3,405 Women
W/H	to systolic pressure	$\approx 0.196 \pm 0.011$	$\text{and } 0.262 \pm 0.023$
W/H <sup>2</sup>	to systolic pressure	$\approx 0.183 \pm 0.022$	$\text{and } 0.256 \pm 0.020$
W/H <sup>3</sup>	to systolic pressure	$\approx 0.198 \pm 0.022$	$\text{and } 0.275 \pm 0.023$
$2\sqrt{W}/H$	to systolic pressure	$\approx 0.171 \pm 0.023$	$\text{and } 0.283 \pm 0.022$
$3\sqrt{W}/H$	to systolic pressure	$\approx 0.190 \pm 0.022$	$\text{and } 0.286 \pm 0.023$

Using the formula  $\sigma_D = \sqrt{\sigma_1^2 + \sigma_2^2 - 2r\sigma_1\sigma_2}$ , the difference between any two of these correlations would have to be at least 0.062 in order to be significant. The actual difference in no

## THE PONDERAL INDEX DISTRIBUTION

The distribution of ponderal index among 7,478 males and 2,325 females shows that the ratio ranges from 1.5 in extremely lightweight persons to over 3.7 in extremely heavyweight persons. The mean male ponderal index was 2.3, with a slightly lower mode at 2.2. A standard deviation of 0.3 showed that the bulk of the males was grouped in the range from 2.0 to 2.6. The female distribution showed a mean ponderal index slightly lower than that of males, at 2.2, with a mode at 2.0; the standard deviation was 0.4, which indicates that the bulk of the females was in the range from 1.8 to 2.6.

By clinical impression and comparison with standard tables it was decided to divide the group tentatively into lightweights (a ponderal index up to 2.0), mediumweights (a ponderal index from 2.0 to 2.4), and heavyweights (a ponderal index of 2.5 and over). These groups merge one into the other; in the original statistical analysis much finer subdivisions were used. This division classed 17 per cent of the males and 38 per cent of the females as lightweight,\* 57 per cent males and 41 per cent females as mediumweight, and 26 per cent males and 21 per cent females as heavyweight.

TABLE I\*  
THE RELATIONSHIP OF AGE TO PONDERAL INDEX

AGE GROUPS	UNDER 20	20-29	30-39	40-49	50-59	60 AND OVER	ALL AGES
<i>7,478 Males</i>							
No. of cases	113	1652	2387	1967	943	416	7478
Mean ponderal index	1.96	2.31	2.32	2.37	2.42	2.22	2.30
Mode ponderal index	2.04	2.06	2.25	2.45	2.45	2.25	2.25
Per cent lightweight	51	26	16	12	11	12	17
Per cent heavyweight	6	13	25	35	36	30	27
<i>2,325 Females</i>							
No. of cases	46	595	797	563	232	92	2325
Mean ponderal index	1.95	1.98	2.16	2.28	2.48	2.28	2.10
Mode ponderal index	1.85	1.85	2.05	2.25	2.36	2.17	1.95
Per cent lightweight	72	63	38	20	21	21	38
Per cent heavyweight	6	7	18	33	40	27	21

\*All tables in this paper are condensed from detailed correlation tables. The statistical measures have been expressed to the nearest unit.

With an increase in age the ponderal index increases for both male and female groups (Table I). The twenty-year-old males have a mean ponderal index of 1.96, which steadily rises as the age increases until the peak ponderal index of 2.42 is reached toward the end of the fifth decade. Note that while only 6 per cent of the very young men are heavyweight, fully 35 per cent of the men in the age groups 40 to 60 are heavyweight. The female group shows a similar tendency. Starting at an index of 1.95, comparatively the same as

*Continued from page 809.*

instance exceeds 0.027 in the men and 0.030 in the women. Therefore, for the purposes of this study, the complications inherent in the modifications of the weight-height index can add very little to the value of the index. The weight-height index is used in this paper because it is the simplest and easiest to understand.

Some authors use the sitting height instead of the standing height measure. However, the sitting height measurement is not generally taken by the physician; it is unfamiliar and demands more rigid standardization. At any rate, the statistical difference between the two measures is said to be slight.

\*The fact that the females were a slightly younger group would modify the difference somewhat.

that of males, the female group gains weight more slowly to reach a peak ponderal index in the sixth decade. While only 6 per cent of the young females are heavyweight, in the 50 to 60 year age group 40 per cent of all females are in the heavyweight class. This increase should *not* be interpreted as a physiologic accretion of weight beyond the third decade. A yearly increase in weight does not take place in the physically active farmer group where obesity is relatively rare,<sup>28</sup> nor is it the rule among primitive peoples<sup>29</sup> where

### THE INCREASE OF WEIGHT WITH AGE IN DIFFERENT PRESSURE GROUPS

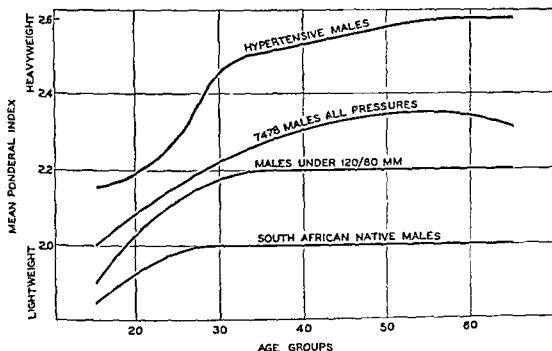


Fig. 1.—In a group of physically active South African natives (30) the maximum weight was reached before the thirtieth year and remained constant throughout adult life. In a civilized group of males, those with low blood pressure followed a similar weight curve, while those with high pressure continued to gain weight over the entire life span.

there is no opportunity for sedentary living. For example, in a study of South African natives, Brodie<sup>30</sup> observed an increase in weight up to age 25, after which the average weight remained at the 135 pound level. These natives were physically active. Only among a sedentary group is there an interference with the normal physiologic processes regulating body weight (Fig. 1). By this interference with the regulation of body weight, the span of life is shortened. The effects of a yearly increase in weight are lucidly illustrated by recent mortality studies.<sup>31, 32</sup> With every increase in weight, beginning with below average levels, there is a progressive increase in gross mortality. That this overweight mortality soon reaches alarming proportions has long been known.<sup>33-35</sup> The high mortality of the obese explains the decrease in mean ponderal index after the fiftieth year, and thus we may surmise that relatively few heavyweights live long enough to be included in the older age groups. Although the peak of overweight in the female occurs about 10 years later than in the male, overweight in the female is accompanied by the same relative mortality tendencies.<sup>36</sup>

### THE RELATION OF PONDERAL INDEX TO SYSTOLIC BLOOD PRESSURE

With an increase in relative obesity, as measured by ponderal index, there was a decided steplike increase in systolic blood pressure. The modal pressure

TABLE II  
THE RELATION OF PONDERAL INDEX TO BLOOD PRESSURE

PONDERAL INDEX	LIGHTWEIGHT			MEDIUMWEIGHT			HEAVYWEIGHT			ALL WEIGHTS	CORRELATION DATA	
	UNDER 1.70	1.70-1.99	2.00-2.29	2.30-2.49	2.50-2.69	2.70-2.99	3.00 & OVER					
1,478 MALES SYSTOLIC PRESSURE	Under 110 mm.	39	369	644	297	183	85	20	1,637	PONDERAL INDEX Mean ----- $2.31 \pm 0.004$ -- $121.0 \pm 0.20$ Std. Dev. -- $0.328$ ----- $17.3$ Skewness -- $0.27$ ----- $0.29$ Coef. Var. -- $14.20$ ----- $14.34$ Coef. Corr. -- $r = 0.19 \pm 0.01$ Corr. Ratio. -- $\eta = 0.21$ Test Lin. -- $\xi = 0.006 \pm 0.002$	PONDERAL INDEX Mean ----- $2.31 \pm 0.004$ -- $121.0 \pm 0.20$ Std. Dev. -- $0.328$ ----- $17.3$ Skewness -- $0.27$ ----- $0.29$ Coef. Var. -- $14.20$ ----- $14.34$ Coef. Corr. -- $r = 0.19 \pm 0.01$ Corr. Ratio. -- $\eta = 0.21$ Test Lin. -- $\xi = 0.006 \pm 0.002$	
	110-119 mm.	20	363	845	478	282	160	52	2,200			
	120-139 mm.	26	384	974	585	497	299	70	2,835			
	140 mm. and over	6	66	218	185	140	131	60	806			
	Total	91	1,182	2,681	1,545	1,102	675	202	7,478			
1,478 FEMALES SYSTOLIC PRESSURE	Under 110 mm.	116	117	119	122	124	127	130	121	PONDERAL INDEX Mean ----- $2.19 \pm 0.008$ -- $117.0 \pm 0.41$ Std. Dev. -- $0.402$ ----- $19.87$ Skewness -- $0.597$ ----- $0.48$ Coef. Var. -- $18.36$ ----- $16.98$ Coef. Corr. -- $r = 0.260 \pm 0.019$ Corr. Ratio. -- $\eta = 0.281$ Test Lin. -- $\xi = 0.021 \pm 0.006$	PONDERAL INDEX Mean ----- $2.19 \pm 0.008$ -- $117.0 \pm 0.41$ Std. Dev. -- $0.402$ ----- $19.87$ Skewness -- $0.597$ ----- $0.48$ Coef. Var. -- $18.36$ ----- $16.98$ Coef. Corr. -- $r = 0.260 \pm 0.019$ Corr. Ratio. -- $\eta = 0.281$ Test Lin. -- $\xi = 0.021 \pm 0.006$	
	110-119 mm.	106	115	117	115	119	124	117	116			
	120-139 mm.	43	31	24	19	17	13	10	22			
	140 mm. and over	65	62	56	50	42	36	36	51			
	Total	7	6	8	12	13	19	30	11			
1,478 MALES DIASTOLIC PRESSURE	Under 60 mm.	15	109	150	52	17	11	2	356	PONDERAL INDEX Mean ----- $2.31 \pm 0.004$ -- $74.4 \pm 0.12$ Std. Dev. -- $0.328$ ----- $10.49$ Skewness -- $0.27$ ----- $0.31$ Coef. Var. -- $14.20$ ----- $14.10$ Coef. Corr. -- $r = 0.274 \pm 0.011$ Corr. Ratio. -- $\eta = 0.285$ Test Lin. -- $\xi = 0.006 \pm 0.002$	PONDERAL INDEX Mean ----- $2.31 \pm 0.004$ -- $74.4 \pm 0.12$ Std. Dev. -- $0.328$ ----- $10.49$ Skewness -- $0.27$ ----- $0.31$ Coef. Var. -- $14.20$ ----- $14.10$ Coef. Corr. -- $r = 0.274 \pm 0.011$ Corr. Ratio. -- $\eta = 0.285$ Test Lin. -- $\xi = 0.006 \pm 0.002$	
	60-79 mm.	57	887	1,962	1,024	644	335	91	5,000			
	80-89 mm.	14	153	453	349	327	221	71	1,588			
	90 mm. and over	5	33	110	120	114	108	38	534			
	Total	91	1,182	2,681	1,545	1,102	675	202	7,478			
1,478 FEMALES DIASTOLIC PRESSURE	Under 60 mm.	69	71	73	75	77	80	81	74	PONDERAL INDEX Mean ----- $2.19 \pm 0.008$ -- $71.5 \pm 0.24$ Std. Dev. -- $0.402$ ----- $11.36$ Skewness -- $0.597$ ----- $0.11$ Coef. Var. -- $18.36$ ----- $15.89$ Coef. Corr. -- $r = 0.260 \pm 0.019$ Corr. Ratio. -- $\eta = 0.281$ Test Lin. -- $\xi = 0.021 \pm 0.006$	PONDERAL INDEX Mean ----- $2.19 \pm 0.008$ -- $71.5 \pm 0.24$ Std. Dev. -- $0.402$ ----- $11.36$ Skewness -- $0.597$ ----- $0.11$ Coef. Var. -- $18.36$ ----- $15.89$ Coef. Corr. -- $r = 0.260 \pm 0.019$ Corr. Ratio. -- $\eta = 0.281$ Test Lin. -- $\xi = 0.021 \pm 0.006$	
	60-79 mm.	30	119	73	29	12	17	9	294			
	80-89 mm.	106	528	430	168	126	95	43	1,505			
	90 mm. and over	14	72	121	50	50	53	24	384			
	Total	153	737	671	272	215	182	95	2,325			

### THE INCIDENCE OF OBESITY IN LOW AND HIGH SYSTOLIC PRESSURE GROUPS

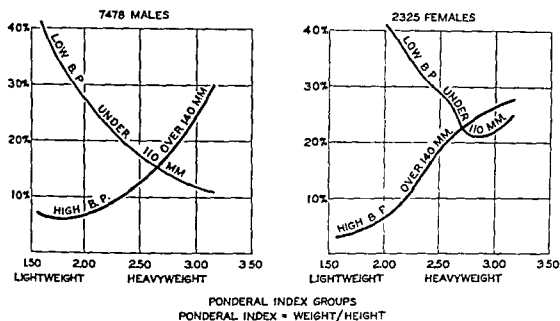


Fig. 2.—The incidence of high pressure is less frequent among the lightweights and increases in frequency as weight increases. On the other hand low blood pressure is most frequent among the lightweights and sharply decreases with increase in weight.

### THE RELATION OF WEIGHT TO BLOOD PRESSURE PERCENT OF ACTUAL TO EXPECTED INCIDENCE

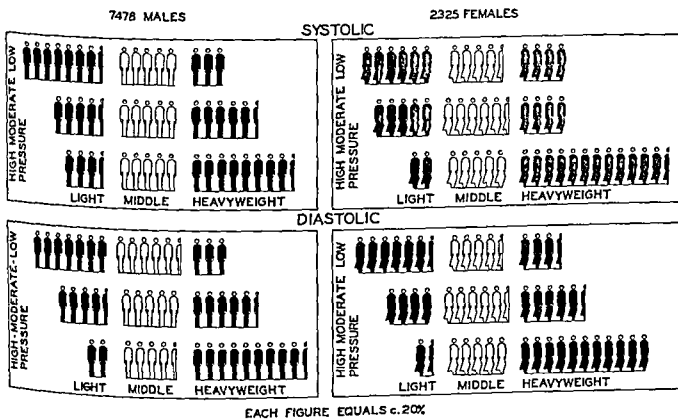


Fig. 3.—High pressures are more common among the heavyweights and low pressures are more common among the lightweights.

increased with an increase in relative obesity, though not as markedly as did the mean.\* This indicates that there is not a pound-for-pound, millimeter-for-millimeter rise, but instead, an increasing incidence of high blood pressures with each increase in relative obesity (Table II). That this is more striking in the females is to be expected because of the very low pressures we observed in young females at the age when we expect them to have a relatively low weight. Lightweight females have lower pressures while heavyweight females have the same systolic pressures that are observed in men of similar weight.

The relative incidence of low and high pressures in the various weight categories shows this relationship clearly (Fig. 2). Note the high incidence of low pressures (40 per cent) in the lightweight males. This incidence sharply decreases with each increase in weight until among the obese males only one-tenth of the group has low pressures. Conversely, in the incidence of high pressures, the very opposite tendency is apparent. Starting with an incidence of only 7 per cent of high pressures among the lightweight males, there is a slow accumulation of high pressures in the middleweight group and then an accelerated rise to a 30 per cent incidence of high pressures among the obese males.

In the female group the tendency is similar. The lightweight females have a high incidence of low pressures and a low incidence of high pressures, while the heavyweight females have a lower incidence of low pressures (though not as strikingly low as the males) and a higher incidence of high pressures. This crossing of the incidence curves of low and high pressures, as pictured in Fig. 2, is a dramatic illustration of the effects of weight on blood pressure.

TABLE III

PER CENT ACTUAL TO EXPECTED RATIO PONDERAL INDEX TO SYSTOLIC BLOOD PRESSURE

	7,478 MALES		2,325 FEMALES	
	LIGHTWEIGHTS (UNDER 2.0)	HEAVYWEIGHTS (OVER 2.4)	LIGHTWEIGHTS (UNDER 2.0)	HEAVYWEIGHTS (OVER 2.4)
Low pressure (Under 110 mm.)	145	68	135	79
High pressure (140 mm. and over)	55	155	36	240

The per cent actual to expected ratio shows clearly the dichotomy of underweight-low pressures and overweight-high pressures (Table III).† There is almost a 2.5 to 1 chance of the hypertensive person being overweight instead

\*In the male group the lightweights had a mean blood pressure of approximately 117 mm. as compared with 121 mm. in the mediumweights. Among the heavyweights the mean systolic pressure was 124 mm. in the least heavy group, rising to over 150 mm. in the very obese. The standard errors of these means were small, thus indicating a significant difference between the blood pressures of the lightweights, mediumweights, and heavyweights. For example, using the formula  $3\sqrt{(sx_1)^2 + (sx_2)^2}$ , the difference between the lightweight pressure ( $116.5 \pm 0.41$ ) and the mediumweight pressure ( $120.9 \pm 0.23$ ) would need be only 1.5 mm. to be significant; the actual difference is 4.3 mm.

†The per cent actual to expected ratio is very simply calculated and is useful in picturing the relative effect of the extremes of one array upon the dispersion of another correlated array. We have used this technique to show the unusual influence of weight on blood pressure. For example, if, as in any random group, we find 22 blood pressures under 110 mm. to every 11 blood pressures over 140 mm., and if there were no correlation of weight to blood pressure, we should find the same ratio, 22 to 11, in any group of overweight. This is not the case; there are actually only 15 instead of 22 overweights with systolic pressures under 110 mm., and 17 instead of 11 overweights with pressures over 140 mm. This gives a per cent actual over expected incidence of 15/22, or only 68 per cent, of expected low pressures, as compared with 17/11, or 155 per cent, of expected high pressures among our heavyweight group. Simply stated, the expectancy of hypertension among heavyweights is almost 2.5 to 1.

of underweight, and the underweight person has the same chance of having a low pressure. The per cent actual to expected ratio shows the female high pressures to be even more strikingly associated with obesity than is the case with the males. Thus we find a 6 to 1 chance of the hypertensive female being overweight, while the lightweight female has almost a 4 to 1 chance of having a low pressure (Fig. 3).

It might be argued by some that the factor of age has its effect on these figures because the higher pressures and the overweights are found in the older ages, while the underweights and lower pressures are found more generally in younger persons. This criticism is partially circumvented by plotting the correlation in each age group separately and constructing this multiple correlation in the form of a three-dimensional graph, as in Fig. 4. Each row

### THE RELATION OF PONDERAL INDEX TO SYSTOLIC PRESSURE IN SPECIFIC AGE GROUPS

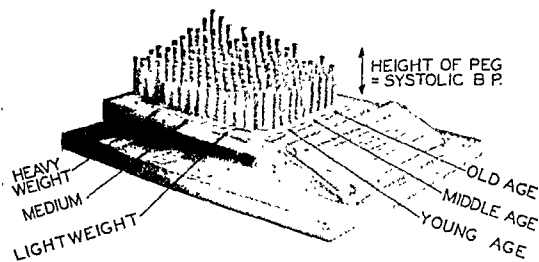


Fig. 4.—See description in text. From a model exhibited at the St. Louis Sessions of the American Medical Association, 1939

of pegs represents an age group, from 15 to 19 years on the extreme left to 70 years and over on the extreme right. Each age group is broken down into ponderal index groups, 1.5 (the very thin persons) in the extreme front to 3.0 (the very obese) in the extreme rear. The height of the peg represents the height of the average systolic pressure for each specific age-ponderal index group.

From this multiple correlation graph we learn that in each male age group the average systolic pressure tends to rise with an increased ponderal index and that the more noticeable elevations come in the older age groups. This cannot be interpreted superficially as a pound-for-pound, millimeter-for-millimeter rise. It is cited, rather, as evidence that hypertensive persons fall most commonly in the overweight groups. The overweight groups in the older ages are more intensively affected by overweight-hypertensive males than are the young ages (Table IV). In the relatively few cases where hypertension does fall in an underweight group, it is almost always in middle-old age. In the female age groups the relationship is not as apparent. The very young and



the very old females do not follow this order. The paucity of subjects over 60 years of age lends some doubt to any interpretation of the higher pressures among the lightweight females; a few unusual subjects can easily distort the mean figure. However, an adequate explanation of this discrepancy has not yet been worked out.

TABLE IV  
PONDERAL INDEX TO SYSTOLIC BLOOD PRESSURE AT VARIOUS AGES

PONDERAL INDEX		UNDER 30 YEARS		AGES 30-39		AGES 40-49		AGES 50-59		AGES 60 AND OVER	
		M	F	M	F	M	F	M	F	M	F
Light Weight	1.7 and under	116	109	112	112	113	111	121	127	139	154
	1.8	116	110	115	111	117	116	115	118	123	152
	1.9	117	109	114	111	119	116	125	126	130	153
Medium Weight	2.0	118	112	117	112	118	119	122	120	128	149
	2.1	119	111	117	111	118	118	124	128	128	142
	2.2	120	111	116	115	120	118	123	125	134	138
	2.3	119	114	118	114	122	122	128	133	144	148
	2.4	121	115	118	118	120	124	131	133	134	143
Heavy Weight	2.5	121	113	119	115	117	126	130	132	138	128
	2.6	120	111	121	113	125	131	126	127	131	146
	2.7	121	105	124	119	125	130	133	130	138	118
	2.8	124	111	122	116	124	126	140	134	142	113
	2.9	122	-	121	123	131	131	132	144	135	133
	3.0 and over	119	110	124	122	129	129	137	138	147	149
Total cases		1,748	635	2,387	797	1,967	563	943	232	391	84

If the incidence of high systolic pressures among the heavyweights, the lightweights, and the total male group is compared in each age-group (Fig. 5), the heavyweights show an accelerating increase which tapers off at the older ages. The acceleration of incidence of high pressures in the total group lags somewhat behind the heavyweight group, but at the older ages tends to approach the heavyweight curve. While there is a rise in the incidence of high pressures among the underweights, the curve falls away from the total group curve as the age increases, and is not as apparent as in the heavyweights.

In order to visualize more clearly the effect of age, we have broken the pressures into two classifications: systolic pressures under 110 mm. and pressures over 140 mm., and each of these divisions was further broken down into two weight classes, light and heavy, for each age group. In every age group except the last the lightweight men and women show a higher proportion of low pressure than high pressure (Table V). However, as the group becomes older the ratio between low and high pressures decreases. The heavyweight men and women show a greater incidence of low pressure than high pressures up to the forty-fifth year (though much less than is found among lightweights). After age 45 this ratio is entirely reversed, there being more high pressures than low pressures among heavyweight men and women. In every age group there is a higher percentage of high pressures among the heavyweight than among lightweight men. In women this is also true except in the very young and the very old.

When the incidence of high and low blood pressures in their divided weight categories is compared on the basis of the per cent actual to expected incidence, we see very plainly the tendency toward a heavyweight-high pressure and lightweight-low pressure grouping (Table VI). Note that in each age group light-

weights show a tendency toward low pressure, and heavyweights tend to congregate in the high pressure group. Contrariwise, heavyweights show a very low ratio of low pressures and a high ratio of high pressures. Further, with an increase in age the lightweight-low pressure group shows an increased ratio. The fact that the heavyweight-high pressure ratio decreases as the group becomes older must be interpreted in the light of its relationship to the total group. While the actual incidence of high pressures increases with age among the heavyweights, the incidence of high pressures in the total group also increases, and in old age approaches the incidence among the heavyweights. Thus, the ratio

### INCIDENCE OF HYPERTENSION IN OBESE AND IN UNDERWEIGHT MALES

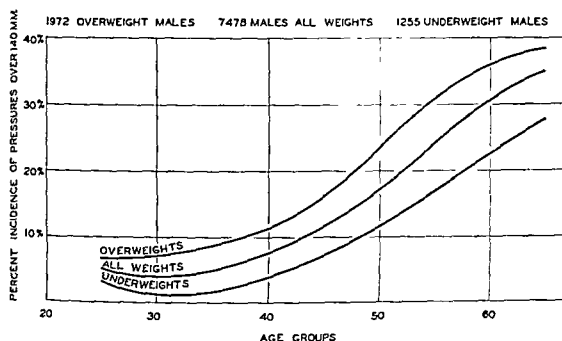


Fig. 5.—In all age groups overweight individuals show a higher incidence of hypertensive pressures.

between the two incidences will decrease. Conversely, the lightweight-high pressure incidence increment falls away from the total group curve, and the ratio between the two increases somewhat. Again, the very old female group does not conform to the general low weight-low pressure tendency—an unexplained discrepancy. In the other age groups hypertension falls more predominantly among the overweights. But it is important to call attention to the decreased correlation between obesity and hypertension as age progresses, a finding contrary to what one would expect.

### THE RELATION OF PONDERAL INDEX TO DIASTOLIC PRESSURE

The male diastolic pressure, as did the systolic pressure, showed a definite increase in vascular tension with an increase in relative obesity (Table II). In the very lightweight males the modal diastolic pressure was around 70 mm. and rose fully 10 mm. as the relative obesity index rose to the heavyweight class. Whereas about 16 per cent of the lightweight males had pressures under 60 mm., less than 1 per cent of the heavyweight males had low pressures; and whereas only 5 per cent of the lightweight males had high pressures, fully 19 per cent of the heavyweight males had pressures over 90 mm.

TABLE V  
PERCENTAGE INCIDENCE OF LOW AND HIGH SYSTOLIC PRESSURE AMONG LIGHTWEIGHT AND HEAVYWEIGHT MEN AND WOMEN

AGE WEIGHT GROUP	20-24		25-29		30-34		35-39		40-44		45-49		50-54		55-59		60 AND OVER	
	LIGHT	HEAVY	L	H	L	H	L	H	L	H	L	H	L	H	L	H	L	H
Men																		
Under 110 mm.	23	23	28	14	37	16	37	17	33	17	33	14	35	12	36	10	28	8
140 mm. and over	6	9	2	6	0	9	3	9	5	12	10	20	13	32	23	30	28	38
Total number	170	44	261	175	234	249	158	342	129	356	105	342	68	197	39	141	40	112
Women																		
Under 110 mm.	58	50	52	40	47	35	46	38	46	22	41	17	23	18	29	9	9	13
140 mm. and over	0	0	1	0	1	8	2	10	4	23	7	27	18	24	23	45	53	31
Total number	222	14	309	45	270	83	174	122	96	150	58	144	39	74	24	53	11	16

TABLE VI  
PER CENT ACTUAL TO EXPECTED INCIDENCE  
PONDERAL INDEX TO SYSTOLIC BLOOD PRESSURE AT VARIOUS AGES

	7,478 MALES		2,325 FEMALES	
	LIGHTWEIGHT	HEAVYWEIGHT	LIGHTWEIGHT	HEAVYWEIGHT
Under 30 years	Low pressure High pressure	124 60	71 120	106 68
Ages 30-39	Low pressure High pressure	148 40	68 180	104 25
Ages 40-49	Low pressure High pressure	143 58	65 133	133 26
Ages 50-59	Low pressure High pressure	200 74	61 135	145 68
60 years and over	Low pressure High pressure	217 80	67 169	140 146
				73 200 93 300 70 159 71 129 44 72

The female group showed a similar distribution. The lightweight females had a diastolic pressure of about 66 mm., which rose fully 10 mm. in the overweight group. While 20 per cent of the lightweight females had pressures under 60 mm., only about 8 per cent of the overweights had low pressures. Conversely, while only 2 per cent of the lightweight females had pressures over 90 mm., fully 20 per cent of the obese had high pressures.

TABLE VII  
PER CENT ACTUAL TO EXPECTED RATIO  
PONDERAL INDEX TO DIASTOLIC BLOOD PRESSURE

	7478 MALES		2325 FEMALES	
	LIGHTWEIGHT	HEAVYWEIGHT	LIGHTWEIGHT	HEAVYWEIGHT
Low pressure (under 70 mm.)	143	57	130	68
High pressure (90 mm. and over)	43	186	33	217

The per cent actual to expected ratio illustrates this relationship more clearly (Table VII). Of the lightweight males, 143 per cent of the expected will have a low pressure, while only 43 per cent will have a high pressure. Conversely, of the heavyweight males, 57 per cent of the expected will have a low pressure, while 186 per cent will have a high pressure. The female group is similarly inclined. The lightweight-low pressure group shows 130 per cent of the expected incidence, and the lightweight-high pressure group shows only 33 per cent of the incidence expected. Conversely, the heavyweight-low pressure group is only 68 per cent of the expected, and the heavyweight-high pressure group shows an incidence double that of expected. Thus, in both males and females a light weight usually is associated with low diastolic pressure and a heavy weight with high diastolic pressure.

TABLE VIII  
PONDERAL INDEX TO DIASTOLIC BLOOD PRESSURE

PONDERAL INDEX		UNDER 30 YEARS		AGES 30-39		AGES 40-49		AGES 50-59		AGES 60 AND OVER	
		M	F	M	F	M	F	M	F	M	F
Light Weight	1.7 and under	69	67	70	69	72	68	74	78	76	75
	1.8	70	68	70	69	73	70	70	70	71	87
	1.9	70	65	70	69	74	72	76	76	71	78
Medium Weight	2.0	71	67	71	72	73	74	74	76	73	83
	2.1	70	68	72	70	74	75	76	75	75	82
	2.2	72	67	72	72	75	74	74	73	78	78
	2.3	72	70	73	70	77	74	78	78	80	84
	2.4	75	69	74	73	76	75	78	77	77	84
Heavy Weight	2.5	74	69	76	75	77	78	80	81	81	76
	2.6	76	67	77	68	79	80	77	76	78	84
	2.7	76	65	78	73	80	77	83	76	81	73
	2.8	76	71	78	76	80	76	84	77	81	73
	2.9	76	-	79	74	83	80	81	82	71	78
	3.0 and over	76	71	80	76	82	79	81	82	86	86
Total cases		1,748	635	2,386	797	1,967	563	943	232	391	84

As with the systolic pressures, it has been argued that the age factor has its effect on these conclusions, because with an increase in age we find an increase in both weight and pressure. To circumvent this criticism the ponderal index-pressure correlation was carried out in each age group where this tend-

ency of pressure to rise as weight increases was again noted (Table VIII). Again, in the aged females either the low frequency has distorted the sample or there is another factor operating which we have not as yet explained. Except for this irregularity, no matter what the age, the heavyweight person is more liable to a high diastolic pressure than either the mediumweight or the lightweight person. This evidence bears out our clinical experience: that hypertension is definitely a hazard among overweights and conversely, even more strikingly, overweight is a hazard of hypertension.

#### DISCUSSION

We have investigated the relationship between weight and blood pressure in a group of 10,883 individuals, residents of an urban region. An increase in weight was found to be concomitant with an increase in blood pressure. However, weight alone was found to be a poor indication of excessive fat storage because of the wide variations of obesity possible in identical weight groups. By taking height into consideration a more accurate index of relative obesity is obtained. Most existing studies are lacking in one or more of the essential criteria for a conclusive analysis, which, combined with a lack of standardization, has led to considerable divergence of opinion on the question of weight and blood pressure. In our sample we found ponderal index, an accurate measure of obesity, to vary directly with both systolic and diastolic pressure in such a manner that high pressure tended to congregate among the heavyweights and low pressure tended to be most usual among lightweights.

Since the incidence of obesity and hypertension increases with age in about the same degree, a study of mixed age groups does not measure their intrinsic correlation. Within specific age groups this obesity-hypertension correlation holds true excepting in the oldest age group. Since the degree of correlation increases with age, it is surprising to find that the oldest age group studied fails to follow the expected trend. We cannot give an explanation for this disturbing deviant correlation.

Because hypertension begins at an earlier age than obesity and because of the high incidence of familial hypertension, heredity is probably the first factor in the predisposition toward hypertension. Body build, reflecting the genetic tendency, predisposes the individual to different pressure levels, as preliminary studies indicate. It is essential to separate the obesity factor from the body-build factor. Current literature on hypertension and obesity has completely ignored their interrelation. The interrelation of body build, obesity, and hypertension will be the subject of a subsequent study.<sup>37, 38</sup>

#### CONCLUSIONS

1. In the analysis of 7,478 males and 3,405 females, weight alone was shown to be a poor measure of obesity. Consequently, a ponderal index—weight divided by height—was used as an index of relative overweight.
2. With an increase in age there is an increase in weight, as measured by ponderal index. After the age of 30 this increase in weight is probably a pathologic process.
3. With an increase in ponderal index toward obesity there is a steplike rise in both mean systolic and diastolic blood pressures in the male and female.

4. The modal systolic and diastolic pressures increased with an increase in ponderal index toward heavier weight though not as markedly as did the mean.

5. The per cent actual to expected ratio shows that the obese males have almost three times more systolic hypertension and almost four and one-half times more diastolic hypertension than do the underweight males.

6. The obese females have six times more systolic and diastolic hypertension than do the underweight females

7. The obese males have only one-half as many low systolic pressures and only five-eighths as many low diastolic pressures as do the underweight males.

8. The obese females have only three-eighths as many low systolic pressures and one-half as many low diastolic pressures as do underweight females.

9. In any hypertensive group overweight is proportionately more common than underweight, and, conversely, in any low pressure group underweight is more common than overweight.

10. The per cent actual to expected ratio shows that among obese males high systolic pressures are two and one-half times more common than low pressures, while high diastolic pressures are three times more common than low pressures.

11. Among overweight females there are three times more high systolic and high diastolic pressures than low systolic and low diastolic pressures.

12. Among underweight males there are two and one-half times more low systolic pressures than high systolic pressures, and three and one-half times more low diastolic pressures than high diastolic pressures

13. Among underweight females there are almost four times more low systolic and diastolic pressures than high systolic and diastolic pressures.

14. In every age group (except females over 60 years) there is an increase in mean systolic and diastolic pressures with an increase in weight.

15. Among the overweights under the age of 45 there are more low pressures than high pressures. This ratio between low and high pressures decreases with succeeding decades so that at the age of 45 there is a complete reversal and thereafter there are more high than low pressures.

16. The per cent actual to expected ratio shows that in every age group overweight males and females more often have high pressures than low pressures.

17. In every age group underweight males and females (except females over 60 years) more often have low pressures than high pressures.

18. In every age group hypertensive males and females (except females over 60 years) more often are overweight than underweight.

19. In every age group males and females with low pressure more often are underweight than overweight.

20. In general, it may be said that obesity and hypertension are frequently associated, but judgment as to the significance of this fact must be withheld until the influence of body build on obesity has been appraised.

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## 145 DRUNKEN DRIVERS—A BLOOD AND URINE ALCOHOL STUDY\*

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IN A previous article,<sup>1</sup> we mentioned the fact that about 100 drunken drivers had been observed by this laboratory for alcoholic content of their blood and urine. It is the purpose of this article to present the results of this study.

We had hoped that by this time a much larger number of cases could be reported, but unfortunately, due to noncooperation, the taking of specimens on accused drunken drivers dropped off and practically ceased as a routine procedure.

Of 145 persons, we took both blood and urine specimens on 66. This gave us a chance to observe the accused person when the call to the police station was made to obtain the blood; we were, therefore, able to make a full set of observations on each person. The method used was that of the National Safety Council's Committee on Tests for Intoxication as previously explained.<sup>2</sup> It also gave us an opportunity to note the degree of correlation between the concentration of alcohol in the urine of these subjects and that of their blood.

There were several other advantages, chiefly from the legal standpoint. For example, one may testify repeatedly that the results of urine alcohol tests are, as a rule, close to the results of the blood test. One may state that the results run parallel after the first one and one-half hours, with the urine slightly higher, but in a close definite ratio to the blood. All this is not as definite, nor does it have the legal weight, as does being able to testify from experience with a large number of cases where blood and urine were run simultaneously. This also makes the acceptance of the urine result alone much easier. Another legal advantage to such a procedure is that one is enabled to testify from one's own experience that there is a close relationship between the results of the analysis and the observations.

Of the 145 persons, 54 had urine tests, 25 had blood tests, and 66 had both urine and blood tests.

Fig. 1 shows the correlation between the 66 blood and urine results. The first point that strikes one's attention is the peculiar bunching of the results in a rough square bounded by 0.15 to 0.25 per cent in the blood concentrations, and by 0.2 to 0.3 per cent in the urine. This peculiarity is only apparent as it becomes noticed within a few weeks of experience in running the tests. One discovers as a consequence, if the Heise method is used, that he may discard all but one dilution of the distillate as a matter of routine, and use only the one that covers the 2 to 3 mg. range in the urine and the 1.5 to 2.5 mg. range

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in the blood. He is enabled to do this because the majority of drunken drivers fall in this class and an unknown specimen may usually be expected to contain this amount of alcohol.

A few words of explanation will make the reason for this clear. A person with a blood alcohol below 1.2 mg. per c.c. and a urine alcohol below 1.5 mg. is, as a rule, not intoxicated enough to make the effect on his driving grossly noticeable, and as a consequence, does not usually run afoul of the law unless he has an accident. Conversely, a person with over 1.5 mg. of alcohol per c.c. in his blood and over 2.0 mg. per c.c. in his urine is, as a rule, intoxicated enough to interfere with his driving to make it noticeable and invite arrest.

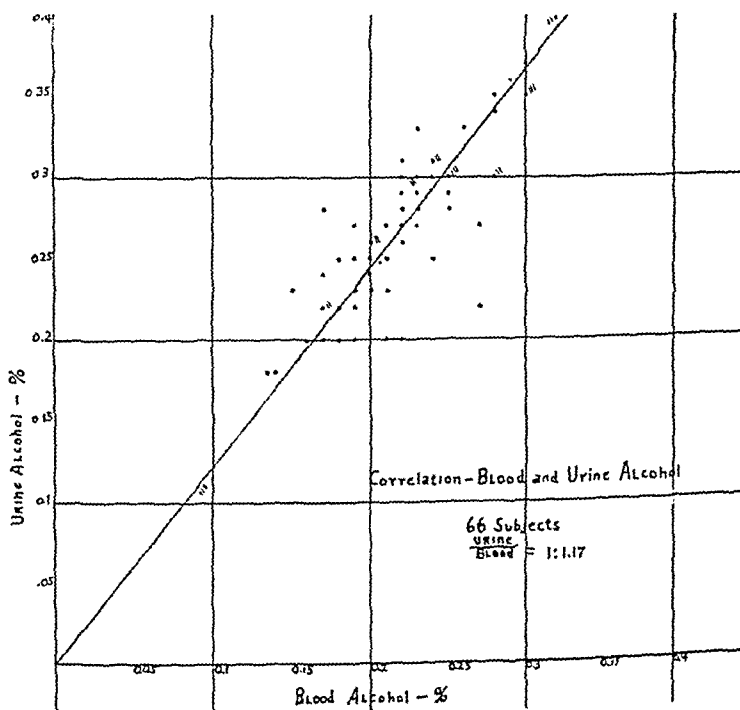


Fig. 1.

The paucity of results over 3.0 mg. per c.c. in the blood and 3.5 mg. per c.c. in the urine is likewise explainable. A person with that much alcohol in his body is nearly always too intoxicated to manipulate the controls of a car, and hence does not appear to any extent among a large group of drunken driver arrests. This is obvious when it is realized that a concentration of 3.0 mg. per c.c. in the blood of a 150 pound man is equivalent to about 12 ounces of whiskey. When one approaches one pint of whiskey, there are few, if any, individuals who will escape serious intoxication.

Table I shows the correlation of the concentration of alcohol in the urine with the degree of intoxication of 120 consecutive cases. The results, as shown by the table, agree well with those reported from other sources; that is, a majority of the persons fall in the 0.2 to 0.3 per cent class, and of these, 56 per cent were moderately intoxicated. By moderately, we mean the so-called

clinical stage of confusion. A representative person in this class is about 40 years old, of average weight, who has obtained his alcohol from whiskey, gin, spiked beer, or pop. He was either reported driving on the wrong side of the street, probably fast and in a manner irregular enough to draw attention; or sideswiped another car or collided with the rear end of one. Sometimes instead, he collides with the curb in rounding a corner, or runs over the curb and hits a tree, telephone, or light pole. When this same driver is on the highway, he has an accident that usually results in serious injuries or death to someone. In the city the accident is usually a minor one. The reason for the more serious results on the highway is, of course, the greater speed on the highway. He is usually arrested during the week end and usually between the hours of midnight and 4 A.M., and, as a rule not far from a beer tavern.

TABLE I

CORRELATION OF CONCENTRATIONS OF ALCOHOL IN URINE WITH DEGREE OF INTOXICATION OF 120 SUBJECTS

URINE ALCOHOL CONCENTRATION PERCENTAGE	NO. CASES	DEGREE OF INFLUENCE BY PERCENTAGES			
		NOT INFLUENCED	SLIGHTLY INFLUENCED	MODERATELY INFLUENCED	STRONGLY INFLUENCED
0.0 - 0.10	1	1 case			
0.11 - 0.20	18	1 case	67%	27%	
0.21 - 0.30	69	1 case	11%	88%	
0.31 and over	32	1 case	0	94%	2 cases
Total	120	4 cases	16%	80%	2 cases

When brought to the station, he always insists he is not drunk and unwittingly cooperates, sometimes comically, in attempting to prove that he is not. His stock in trade alibi is that he had had two glasses of beer. When examined, his speech is usually moderately thick, his pulse usually rapid, his eyes watery and fairly often bloodshot, his pupils moderately dilated; his face is nearly always flushed and sometimes his hands and forearms as well. He may be in almost any emotional state, from morose stubborn silence to wild hilarity or tears. He walks with a decided, though slight, stagger, and if blindfolded, will always sway to some extent, usually so badly that he will lose his balance entirely. He will almost always willingly agree to the taking of a blood specimen—we have had only two who refused during the two years. His skin sensibility is noticeably deadened—venipuncture is not felt, judging from the lack of the slight finching which a sober adult usually manifests. The result of the urine test in this stage will show about 2.3 to 2.5 mg. of alcohol per c.c., and the blood about 1.8 to 2.0 mg., corresponding in a 150-pound man to about one-half to two-thirds pint of whiskey.

All the symptoms outlined will not necessarily be present in the same person at the same time. For example, the pupils may be widely dilated, with no flush to the face, or vice versa. In other words, it is the degree and quality of those symptoms that are present that is significant, not the total number of symptoms.

The majority of specimens were run by the Heise method. The Harger titration method was used in about two-thirds of the cases to confirm the Heise result.

Our study of the 145 persons covers a period of a little over two years. During this time, constant checking of results against each other and of the methods themselves against known amounts of absolute alcohol was carried out. The two methods gave essentially the same results from the standpoint of checks against known amounts of alcohol.

The results, as shown by both tables, confirm previous results by various authors, pointing to the same conclusion; viz., that within the range of normal physiologic variations in individuals and the results of chance sampling of all classes of drunken drivers, the analysis of all body fluids can be expected to reflect about an 85 to 90 per cent true picture of the clinical effects of the amount of alcohol found.

There seems to be some evidence<sup>5</sup> that occasional persons may be found who do not conform to the correlation between the stages of intoxication and the concentrations of alcohol in the body fluids. The majority of this group (constituting probably 10 to 15 per cent of all people) seem to possess the ability to pull themselves together under the influence of shock of accident, arrest, excitement at the station, questioning, and examining. We have repeatedly seen this occur. We have seen a staggering drunk sober up in fifteen minutes during the course of an examination to the extent that he could walk steadily, only to fall asleep when left alone and then stagger again when aroused. There still seems to be a small percentage, probably 2 to 3 per cent, of this nonconforming group that cannot be explained on this basis. For example, an occasional person is encountered with a blood alcohol concentration of 3 mg. per c.c. or over, with little symptoms of intoxication. Jetter<sup>6</sup> reports one with a blood alcohol concentration of even as high as 4 mg. per c.c., who, according to him, showed little symptoms of intoxication. We saw one with 3 mg. that we were forced to describe as being practically sober.

However, we have gained an impression, which we admit seemingly has no very concrete basis, that as far as police work is concerned in drunken driving cases these persons are just as guilty of drunken driving as the person who staggers visibly. It seems somewhat significant that this kind of person seems to be subject to the same type of accidents as the more visibly affected person.

These occasional discrepancies do not invalidate the significance of the results of the tests in police work. The tests are absolute proof of the amount of unburned alcohol present in the person's body at the time the specimens were obtained, and when we prove that a person had close to a pint of whiskey or half a pint of unburned alcohol in his body at the time of arrest, we are splitting hairs when we quibble over the way he walks. He is just as much a menace as the staggering human being with the same amount in his body. The ability a person has to walk has little connection with his ability to drive a car, and when any person has between 2 and 3 mg. of unburned alcohol coursing through his brain, he is utterly incapable of controlling a car in a safe and sane manner for, in the last analysis, it is the state of a person's brain that determines his fitness to drive a car.

In a future paper we shall present the results of some animal experimentation, together with some experimentation on human beings, and a discussion of the problem of occasional puzzling results in more detail. At present we wish to emphasize that persons who apparently do not conform seem to be rare and little or no concern need be given them.

The law is slow to recognize any new scientific procedure. The states of Maine, Indiana, and Illinois have recently rewritten their laws pertaining to drunken drivers, incorporating the results of blood and urine tests as legal evidence. To the arguments of those conscientious objectors who make use of the hackneyed "constitutional rights of the individual against self-incrimination" to triumphantly prove that the use of the tests forces a person to incriminate himself, we simply refer them to the excellent discussion of the legal angles of this question by Professor Ladd.<sup>6</sup> The ridiculousness of this assertion is apparent when one realizes that the original and true meaning of this constitutional provision was to protect the individual from being forcibly made to confess something of which he may not be guilty. Obviously, threats, blows, arguments, or physical pain cannot change the concentration of alcohol in a person's blood. The use of this argument of constitutional right then becomes nothing but a means by which a guilty person may escape punishment and an innocent person sometimes falsely found guilty.

We can say in absolute honesty that never, in the course of our experience with these tests, has a person been falsely incriminated from the results of a test. Unfortunately, we have seen entirely too many found not guilty in spite of conclusive proof of guilt.

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## THE RELATION OF LYMPHOCYTES TO THE ACTIVITY OF MYCOBACTERIUM TUBERCULOSIS\*

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THE role of phagocytes in the battle of the body against disease has been a live question in the world of medical sciences for three quarters of a century, and any fact that can be brought to light on this question is of practical as well as scientific interest. Since the lymphocyte is present in all tubercular lesions, and so greatly preponderates or is the only phagocyte present in established tubercular lesions, our attention is necessarily directed toward it. The question as to the relation and mechanism of the relation of the lymphocyte to tubercular infection is a timely and poignant one.

Rather extensive literature exists on the supposed relationship of the lymphocyte to tubercular infection, but it consists largely of clinical observations and observations in connection with clinical procedures, which, while of great value in establishing the existence of a relationship, do not tell us anything of the nature or mechanism of the relationship. What experimental work has been done has in the main had as its object the test of the truth of clinical observation. Some of the experimental work has led its authors to theoretical conclusions at variance with known scientific facts and is, therefore, subject to question. No experimental work, so far as we are able to find, has shed any light on the character of the lymphocyte-tubercular relationship.

Ullom and Craig<sup>1</sup> first reported the fact that in acute cases of tuberculosis a relative excess of neutrophils is found. This observation is supported by the work of Morris and Tan,<sup>2</sup> who found an increase in polymorphonuclear cells and monocytes in active tuberculosis. Ullom and Craig, however, point out: "The actual increase in lymphocytes seems to correspond to the increase of resistance on the part of the organism to the tuberculous infection; further study is required to confirm this deduction." Wright and King,<sup>3</sup> Watkins,<sup>4</sup> and Solis-Cohen and Strickler<sup>5</sup> have studied the relation of lymphocyte counts to the clinical course of tuberculosis and have concluded that when the lymphocyte count is high, the prognosis is good, and that a decreasing number of lymphocytes indicates a decreasing resistance to the infection. Webb and Williams,<sup>6</sup> and Webb, Newman, and Gilbert<sup>7</sup> are in agreement with the above conclusions. Murphy and Ellis,<sup>8</sup> on the basis of the results of experiments in which the lymphopoietic tissue was decreased by x-ray, believe "the lymphocyte plays an important role in the animal's resistance to tuberculosis." Hussey<sup>9</sup> also believes that the lymphocyte plays a part in the resistance to tuberculosis.

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Quite extensive experiments have been carried on in an attempt to show that in tuberculosis there is an inverse relationship between the monocyte and the lymphocyte; i.e., the lymphocyte is an indication of resistance to the course of the disease, whereas an increased number of monocytes indicates a loss of this resistance. The literature in this regard is extensive. Most of this work has been done by Sabin and her co-workers, Sabin,<sup>10</sup> and Sabin and Doan.<sup>11</sup> Medlar,<sup>12</sup> however, feels, "The factor of greatest significance in the leucocytic picture of tuberculous cases is the ratio between the polymorphonuclears and the lymphocytes. The reason for this ratio being of such great importance is that in the tuberculous lesions the polymorphonuclear represent one type (unfavorable) of pathological activity and the lymphocytes another (favorable)." Further, "It is the opinion of the author that the main function of leucocytes is to dispose of products harmful to the normal functioning of living tissue."

The mechanism by which the lymphocyte produces a resistance to tubercle bacteria has been investigated to some extent. Watkins<sup>4</sup> believes that the effectiveness of the lymphocyte in tuberculosis is explained by its elaboration of a lipolytic ferment which dissolves the waxy sheath enclosing the tubercle bacilli. If this is true, it is difficult to see how it could operate in a case of tuberculosis because the tubercle is a nonvascular lesion. Therefore, the bacilli would not be reached by any antibody, and, since few, if any, phagocytes other than lymphocytes are found in tubercles, there would be none to dispose of the tubercle bacilli even if they were stripped of their armor. It could hardly interfere with the growth of the bacilli because of the readiness with which tubercle bacilli grow in lymphatic tissue. Pawlow<sup>13</sup> has reported that lymphocytes will cause a disintegration and clumping of mycobacterium tuberculosis within two and one-half hours at 58° C. If this is the case, it could not be the important factor in lymphatic resistance because such temperatures do not occur in tubercular infections.

The question has been raised as to whether the lymphocytosis in tuberculosis is the manifestation of direct activity on the part of the lymphocyte or whether it is merely correlated with a greater resistance on the part of the individual. Smithburn<sup>14</sup> encouraged a lymphocytosis by injections of embryonic chick extract. He then inoculated rabbits with a virulent strain of tubercle bacillus. It was found that an inverse correlation existed; i.e., animals possessing a high lymphocyte count died sooner than the controls. Commenting on Murphy's work, Smithburn feels that the lymphatic response as indicated (by Murphy) as the cause is in reality the effect of resistance to tuberculosis. Corper<sup>15</sup> also has come to the conclusion that there does not seem to be a relation between the lymphocyte and the course of tuberculosis. The variance of Smithburn's results with almost universal agreement to the contrary might be explained on the basis of the intoxication produced by the chick extract.

#### SUMMARY OF LITERATURE

The observations so far made seem to justify the conclusions: (1) that lymphocytes play an important role in the resistance of the body to the activity of tubercle bacilli; (2) that the magnitude of the lymphocyte count is an index

to the degree of resistance of the body to the activity of tubercle bacilli, and is, therefore, of prognostical value; (3) that the polymonocyte : lymphocyte index varies with the general destructive action of the tubercle bacilli and is, therefore, high in the early acute and late stages of the disease. We have no indication as to the nature of the resistance factor of the lymphocytes, whether this factor is operative on the bacilli or whether the effect is produced in some other way, and if so in what way. The answer to this latter question must solve the paradox that while lymphocytes provide a factor in the resistance against tuberculosis, lymphatic tissue seems the most defenseless tissue in the body to the invasion and activity of tubercle bacilli.

These studies were undertaken to ascertain, if possible, (1) whether a lymphocytosis prior to infection by tubercle bacilli had any effect on the course and duration of the subsequent disease; (2) whether or not lymphocytes had any direct effect on tubercle bacilli as to virulence or in any other way; (3) to check the polymorphonuclear-lymphocyte index of Medlar; (4) granted lymphocytes play a part in the resistance to the action of tubercle bacilli, could any intimation be discovered as to the nature of the resistance factor.

#### MATERIALS AND METHODS

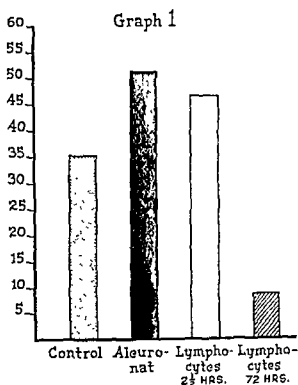
Three-hundred gram guinea pigs were the experimental animals used. They were kept in cages in a well-ventilated room of constant temperature and fed the same food; every precaution was taken to prevent intercurrent infection. The animals were divided into three groups: the first group was used as controls; the second group was untreated; the third group was injected intra-abdominally on three successive days with 5 c.c. of a sterile suspension of aleuronat to produce a lymphocytosis prior to the injection of the bacteria.

Cultures of Jamaica 22 strain of tubercle bacteria were used as the test organism because it is a strain of known high virulence. Two sets of these organisms were prepared. One set was untreated and used for inoculation into the control animals, and those in which a lymphocytosis had been produced. The other set was mixed with suspensions of lymphocytes and incubated for periods varying from two and one-half to seventy-two hours at 37.5° C. The lymphocytes for this purpose were secured by injecting massive doses (15 to 20 c.c. of aleuronat) intra-abdominally into rabbits. A week later the rabbits were injected intra-abdominally with sterile salt solution and killed immediately. The peritoneal fluid was then aspirated by means of a sterile syringe and placed in homeopathic vials. It was found that a high concentration of cells had been obtained, most of which were of the lymphocyte type. Bacteria were added to the lymphocyte suspension and incubated for varying periods of time as indicated.

Suspensions of untreated bacteria in sterile saline were made so that a 3 c.c. dose contained from 14,000 to 20,000 organisms. Three cubic centimeters of this suspension were injected intra-abdominally into each of the control animals, and each of the treated animals five days after the last injection of aleuronat. After incubation as indicated, 3 c.c. of the lymphocyte-bacteria suspension, containing approximately the same number of bacteria per dose as in the doses of untreated bacteria, were injected into animals in the third group.

The survival time of each animal was taken as the criterion of the resistance of the animal to the tubercle bacilli. Autopsies were performed on each animal at death, and the presence of tubercular lesions verified by microscopic study of the organs.

Total and differential counts of 300 cells in each case were made on one animal in the control and aleuronat groups every five days, seven animals were studied almost daily. In addition morphologic and growth studies were made of the bacteria which had been subjected to the lymphocytes in suspension.



#### DISCUSSION

The bacteria which had been placed in the suspension of leucocytes showed no obvious clumping or splitting-up of the bacilli. Dry preparations were made and stained by the Ziehl-Neelsen technique. No obvious changes in morphology were noted. Treated bacteria were seeded on egg-agar slants and resumed their previous growth. No evidence was seen that the lymphocytes had any effect on the morphology or viability of the tubercle bacteria, and no observations were made which would tend to uphold the findings of Pawlow.

The average survival time of the animals in each group is shown in Graph 1. The average survival time of the six control animals was 35.6 days. The longest survival time was 41 days and the shortest was 27 days. The average survival time of the 6 pigs given aleuronat previous to infection was 51.6 days, the range being between 54 and 49 days. The average survival time of 12 pigs given the bacteria-lymphocyte suspension which had been incubated two and one-half hours, was 46.8 days. The longest survival time in the group was 65 days and the shortest was 27 days. Two animals given the bacteria-lymphocyte suspension which had been incubated for seventy-two hours, died in the remarkably short time of nine days. Both were found to have died of tuberculosis infection. The viscera were almost completely covered with tubercles.

It is to be noted that while all of the animals died within the average range of longevity of guinea pigs injected with virulent tubercle bacilli, there was a



relatively longer survival time in the groups in which the tubercle bacilli had been exposed to living lymphocytes. The remarkably short survival time of the animals injected with the bacteria-lymphocyte suspension incubated for seventy-two hours was probably due to the effect of the toxins of the dead lymphocytes in addition to those of the bacteria.

The study of the blood pictures in animals in these experiments is interesting. The total white blood cell counts in the control animals showed an immediate rise which reached its peak on the fifth day and then began to drop steadily until the twenty-fifth day, beyond which no counts were made. The total count at the outset of the experiment was highest in the aleuronat animals, being 16,050, and the actual number of lymphocytes was 9,450. It is seen, however, that an immediate fall took place until the twentieth day, when a slight increase was again noted. A short rise was noted in the lymphocyte-bacteria suspension group at the end of the fifth day, with an immediate drop and then an increase in total number.

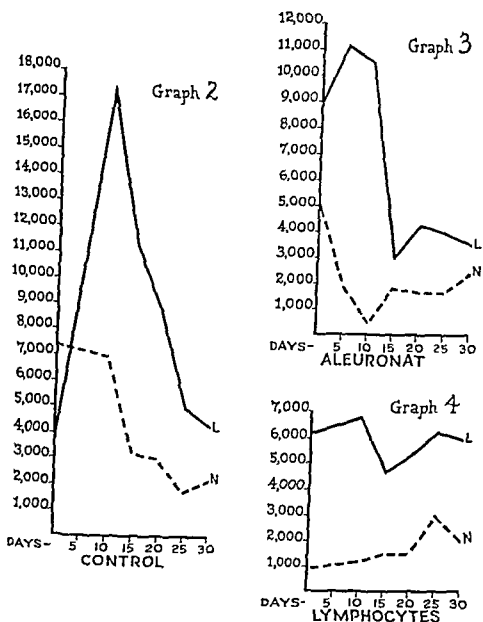
The lymphocyte count and the neutrophile count were calculated in absolute number and plotted to show the relationship of the actual number of lymphocytes to the actual number of neutrophiles. It may be seen that throughout the period of twenty-five days the lymphocytes in all cases remain higher in number than the neutrophiles. The highest number of lymphocytes in all three sets of animals was found on the tenth day, the highest actual number being found in the control animals, but with a more immediate and extensive drop in the number of cells. It is also observed that the polymorphonuclear cells tend to decrease in number during the lymphocyte rise and then begin to maintain a more or less constant level with a slight rise in number. There seems to be an almost reciprocal relationship between the lymphocytes and neutrophiles. Graphs 2 and 3 show the relationship between the actual number of lymphocytes and neutrophiles in one of the control pigs and one of the aleuronat pigs. Graph 4 shows this relationship in one of the lymphocyte pigs. These are the only two studies made on the control and aleuronat sets, whereas seven studies were made in the lymphocyte group; all tend to show the same general relations as in Graph 2. The fundamental nature of the reciprocal relationship between myeloid and lymphoid tissues has been discussed by Wiseman.

A mathematical expression of this relationship may be obtained by dividing the actual number of neutrophiles by the actual number of lymphocytes. It is found that the index for the normal pig is 0.83 (based on values given by Kleinberger and Carl in Kolmer, Boerner, and Garber's *Approved Laboratory Technique*<sup>16</sup>). Thus it may be seen that as the number of neutrophiles increases, the index tends upward; when the tendency is downward, it is due to increased number of lymphocytes, and the greater the resistance of the animal would be expected to be.

The control animal (Graph 2) had a rather high number of neutrophiles at the outset. The index in this case immediately fell to 0.41, and on the fifteenth day fell to 0.29, after which it began to rise and on the twenty-fifth day reached 0.60. The animal died on the thirty-seventh day of infection.

The lymphocyte count increased in the aleuronat animal to such an extent that the index at the outset was 0.62, falling to 0.05 on the fifteenth day and then rising to 0.67 on the twenty-fifth day. This animal lived forty-nine days.

The lymphocyte suspension animals were studied in more detail. In each of the seven the index was quite low, ranging between 0.015 and 0.70 at the outset. It may be further seen that there was less fluctuation in the number of cells at any particular time until shortly before the time of death. These animals, with the exception of the one shown in Graph 4, were studied until the time of death. In each case there was a gradual rise in the number of neutrophils to the time of death, the lowest index being 0.62 and the highest being 2.



It may be further observed that the period of the second week would seem to be, on the basis of these studies, the point of greatest resistance. It is observed that following an initial rise there is a direct downward trend on the part of the lymphocytes, with an upward trend on the part of the polymorphonuclear cells.

Although an increase in monocytes is observed, it does not seem to be as constant as the neutrophilic relationship. There is further difficulty in the identification of monocytes since a "monocytoid" form of lymphocyte appears to be present in the blood of the guinea pig during tuberculous infection.

It is to be further noted that, although Spink<sup>17</sup> finds Kurloff Korper in monocytes during tuberculous infection of the guinea pig, we did not. Most of

the Kurloff Korper (if not all) in this study were found to be in the lymphocytes. A direct relationship between eosinophiles and Kurloff Korper was not noted.

#### CONCLUSIONS

A careful study of the results of these studies seems to warrant the following conclusions:

1. Lymphocytes play an important part in the resistance of the body to the action of tubercle bacilli.

2. This role is due to the activity of the active living lymphocyte. It is not due to any extracellular agglutinin, enzyme, or lysin which acts on the bacilli, but must be due to the lymphocytes disposing of the bacterial toxins, thus robbing the bacteria of their initial assault ammunition. This conclusion is further strengthened by the observation of all pathologists that tubercle bacilli seem to grow more readily in lymphatic rather than any other tissue. In this case the lymphocytes, not attacking the bacteria but disposing of their metabolic products inimical to themselves, allow a more profuse growth.

3. There is a definite lymphocytosis, the magnitude of which varies with the progress of the tubercular disease.

4. The neutrophile-lymphocyte index is highest at the onset of the disease and again at the approaching death of the animal.

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## THE WELCH-LIKE BACILLUS IN HUMAN LIVER\*

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IF A SMALL piece of liver is sectioned aseptically and dropped into the abdominal cavity of a dog or rabbit, death occurs in fifteen to eighteen hours.<sup>1-7, 10</sup> The first recorded observations on this subject were made by Mason and Davidson<sup>1</sup> in 1925, and their autopsy records at that time include the following: "Probably the most marked changes occur in the free liver tissue. It appears to have undergone a complete transformation, there being present no gross characteristics of liver tissue. The color is that of light chocolate while the feel is that of lung tissue, being spongy and gas containing."

It will be observed that they recorded the presence of gas in the autolyzed liver but assumed that such gas was due to the presence of the colon bacillus. However, the gas in the sectioned liver was later shown to be due to the action of Welch-like bacilli.<sup>2, 4, 8-10</sup> Mason and Nau<sup>10</sup> found that the Welch-like organism was present in the central portion of the rabbit's liver and showed that the peripheral portion of the rabbit's liver could be cultured without the presence of the organism. Emulsions of the autolyzing liver containing the organism were injected intravenously into rabbits without any ill effects. If the rabbit was killed a few minutes after the injection and subsequently placed in an incubator, the organism destroyed all soft tissue.

### EXPERIMENTAL

In our present study we have cultured tissue taken from five human livers and in all we have found a Welch-like bacillus. Anaerobic cultures have been made, using pyrogallie acid with 5 per cent sodium hydroxide, but the abundance of gas generated invariably blew out the stoppers; hence we have resorted to covering the tissue with at least 1 inch of paraffin oil instead of using pyrogallie acid. We have collected and measured the gas generated and found that within twenty-four hours it is approximately seven to ten times the volume of liver cultured. Tissue taken from three different livers gave the following:

LIVER Gm.	GAS c.c.	RATIO
6.25	52	8.32
4.6	33	7.2
5.8	61	10.5

Fig. 1 is a photomicrograph of an impression smear of human liver which had been incubated anaerobically for only six hours. It will be observed that red blood cells are present in the smear, and it will be noted that the organisms growing in the human liver show an almost pure culture of the organism. There is considerable variation in the size of the organism, and such variations are com-

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Fig. 1.—Impression smear of human liver incubated anaerobically for six hours.

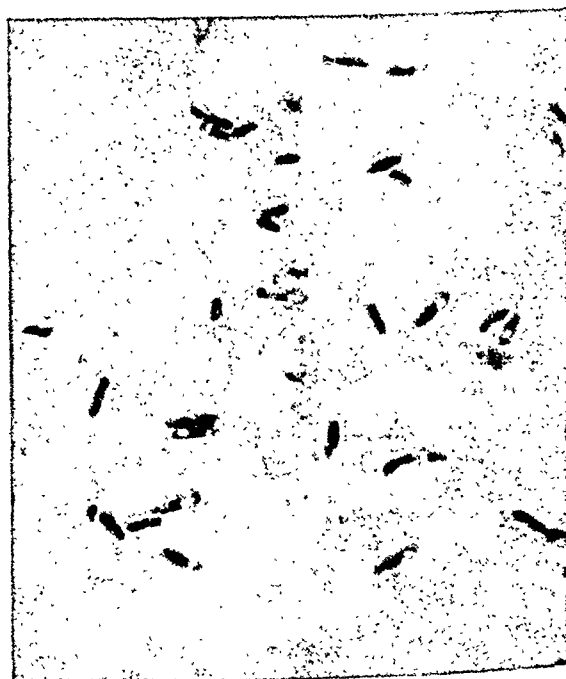


Fig. 2.—Impression smear of human liver incubated anaerobically for twenty-two hours.

monly seen in *B. welchii*. Normally *B. welchii* vary in size from 0.8 to 1.2 micron in diameter, and from 2 to 6 microns in length. It normally occurs singly or in pairs both in culture and in the tissues. Short and nearly coccoid elements are not infrequently seen. The organism shown in Fig. 1 proved to be gram-positive as is also the Welch bacillus.

Fig. 2 is a photomicrograph of an impression smear of human liver which had been incubated anaerobically for twenty-two hours. Again it will be observed that the culture is practically free of all other organisms. The average size of the organism is larger than that shown in the six-hour culture and the spore formation is quite pronounced. Apparently spore formation occurs when the carbohydrate becomes depleted. The maximum activity, measured in terms of gas formation, occurs in approximately fifteen hours and is practically completed at the end of eighteen hours. This again is limited by the amount of carbohydrate present.

We have grown the organism in milk cultures prepared by boiling the milk and cooling it rapidly to expel the oxygen. The milk is then inoculated with the organism obtained from liver culture, covered with an inch of paraffin oil, and heated at 80° C. for twenty minutes to kill all vegetative forms. The organism produces a very marked fermentation of milk.

Three cubic centimeters of the whey obtained from a milk culture were injected intravenously into a rabbit. The rabbit showed marked symptoms within thirty seconds and died within three minutes. It was then incubated for fourteen hours and had all the usual characteristics of a rabbit injected with *B. welchii*. This is known as the Welch-Nuttall rabbit test.

#### SUMMARY AND CONCLUSIONS

We have called this organism the Welch-like bacillus because it has the following characteristics in common with *B. welchii*: (1) It grows under strict anaerobic conditions, (2) is gas-forming, liberating seven to ten volumes of gas from liver tissue, (3) is gram-positive, (4) produces "stormy fermentation of milk," and (5) gives the Welch-Nuttall rabbit test. The bacteriologist, Kendall, considers these last two tests "presumptive tests for *B. welchii*. They are not conclusive but strongly suggestive of the presence of the organism in the material under investigation."

If the response in the human being is similar to that in other animals, it is conceivable that injuries to the human liver, which cause impairment of circulation, may afford a culture medium for Welch-like organisms and thus cause death.

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## THE EFFECT OF REDUCED EVAPORATION ON THE VITAMIN CONTENT OF FRESH VEGETABLES IN REFRIGERATED STORAGE\*

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THERE is ample evidence in the scientific literature that low temperatures help maintain the vitamin content of fresh vegetables, but we have found no report on the effect of reduced evaporation. In this paper evidence will be presented on the effect of storage under increased humidity and decreased air movement, both of which reduce evaporation, on the vitamin content of foods.

### EQUIPMENT

Two types of domestic electric refrigerators were used in this study. Refrigerators A were of conventional construction, cooled by a coil surrounding the ice-cube compartment at the top of the cabinet. As the surface of this coil was small with respect to the cabinet area, it was necessary to maintain a larger temperature differential between the coil and the refrigerator compartment and to keep the coil sufficiently cold to get satisfactory and rapid convection cooling. This caused moisture to condense on the coil, drying the atmosphere of the cabinet, so that ordinarily the relative humidity of the compartment was below 65 per cent.

The compartment of refrigerators H was identical in size to those in refrigerators A but was divided roughly in the middle with a horizontal rubber-sealed glass shelf. The upper section was refrigerated with a coil of the same size as in refrigerators A. In the walls and floor of the lower compartment a long continuous refrigeration coil was imbedded. Because of the large surface of this coil it was necessary to maintain only a slight temperature differential between the coil and the compartment, reducing the condensation so that ordinarily the relative humidity of the lower compartment was 93 per cent or more.

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In all experiments the temperature at the center of each refrigerator at a distance of 25 cm. from the floor was held at about 41° F. This position was 15 cm. below the glass shelf in the H refrigerators and 60 cm. below the ceilings of all the refrigerators. In all experiments comparisons were made between food substances placed in the humid lower compartment of refrigerators II and in corresponding positions in refrigerators A. Temperature, location, and time were substantially the same in all instances except as noted below; essentially only humidity and convection (air movement) were different. Differences in results could, therefore, be ascribed to these two factors.

The rate of evaporation of moisture from foods stored in the A refrigerators averaged some four times that of foods stored in the H refrigerators. Much less moisture was lost by foods placed in covered metal containers on the floor of refrigerators A than when placed on open shelves, because convection was lowered and the humidity of the air in these containers rose to about 93 per cent. Foods in similar vegetable containers and in like positions in refrigerators H lost least moisture because convection was further reduced and the relative humidity was raised above 98 per cent. Evaporation from foods stored in the vegetable containers in A refrigerators was substantially two and one-half times more rapid than in containers in corresponding positions in H refrigerators. This was due in part to a difference of 8° F. in the temperature of the interior of the containers, as in refrigerators A cooling was dependent upon convection from the coils at the top of the refrigerator.

#### MATERIALS AND PROCEDURE

Green vegetables were selected because they are especially active biologically, are sensitive to changes in humidity and air movement, are particularly good sources of the unstable vitamins, and are commonly stored in domestic refrigerators. Chemical, photometric, and biological tests were made to determine the rate of loss of the unstable vitamins A, B<sub>1</sub> (thiamin), and C (ascorbic acid) in these vegetables. Because of the extreme sensitivity of ascorbic acid, and because of the convenience and reliability of the chemical titration procedure by which ascorbic acid is determined, data from measurements of this vitamin predominate. For the chemical and photometric tests, vegetables of the best available quality were secured from local retail markets. For the biological tests on vitamins A and B<sub>1</sub>, green lettuce, and for the biological tests on vitamin C, parsley, were secured daily, freshly picked from the same greenhouse. All tests were made in the late summer and fall of 1938.

Each lot of samples was placed in a small dish in the refrigerators. The dishes were weighed to 0.01 Gm. before and after loading with samples, and again upon removal from storage. Chemical determinations of the vitamin content were then made, and the results compared with data similarly obtained on samples tested before storage. Results are expressed in relation to the original weight of the samples, which were refrigerated for three, four, five, and seven days. Vegetables stored for more than seven days in the A refrigerators were generally unpalatable.



## ASCORBIC ACID, CHEMICAL ASSAY

The amount of ascorbic acid in lettuce was determined by Tillmans' procedure,<sup>1</sup> as modified by Mack and Tressler.<sup>2</sup> In testing vegetables other than lettuce, however, this procedure was departed from to the extent that the pulverized and precipitated cell fragment material was not separated from the extract before titration because it did not interfere with the determinations and it allowed a more rapid determination. Except in one instance, ten or more lots of samples were tested for each position or storage interval, and the loss in ascorbic acid was determined separately for each. From these data the mean averages and variations were calculated. The data from 537 determinations are given in Table I.

TABLE I

EFFECT OF HUMIDITY AND AIR MOVEMENT ON ASCORBIC ACID LOSS  
(Average percentage losses and mean deviation based on original weight.)

POSITION		REFRIGERATORS A	REFRIGERATORS H	REFRIGERATORS H (VEG. CONTAINER)
AVERAGE HUMIDITY		65 per cent	93 per cent	98 per cent
VEGETABLE	DAYS STORED	PERCENTAGE LOSS IN ASCORBIC ACID		
Lettuce	1	26.5 ± 13.3	14.3 ± 16.6	20.4 ± 16.3
	3	55.1 ± 4.1	36.7 ± 10.2	22.4 ± 16.3
	7	48.0 ± 19.4	38.8 ± 19.4	33.7 ± 13.3
	Avg.	43.2 ± 12.5	29.9 ± 15.4	23.5 ± 15.3
Spinach	4.5	77.4 ± 6.8	67.6 ± 13.6	62.4 ± 15.0
	7	82.4 ± 2.6	58.6 ± 10.0	66.4 ± 7.1
	Avg.	79.9 ± 4.7	63.1 ± 11.8	64.4 ± 11.0
Parsley leaves	4	17.4 ± 6.2	8.0 ± 8.9	3.8 ± 10.2
	7	31.8 ± 5.0	21.0 ± 7.9	15.4 ± 7.1
	Avg.	24.6 ± 5.5	14.5 ± 8.4	9.6 ± 8.5
Snap beans	3	30.8 ± 12.8	24.4 ± 12.8	28.2 ± 12.8
	7	50.0 ± 6.4	32.1 ± 6.4	35.9 ± 12.8
	Avg.	40.0 ± 9.6	28.2 ± 9.6	32.0 ± 12.8
Shelled lima beans	4	35.0 ± 10.6	31.5 ± 9.8	19.3 ± 8.3
	7	54.8 ± 4.7	29.2 ± 7.9	28.0 ± 8.7
	7 <sup>a</sup>	39.4 ± 5.5	10.2 ± 8.7	4.7 ± 7.1
	Avg.	43.1 ± 6.9	23.6 ± 8.6	17.3 ± 8.0
Shelled peas	5 <sup>a</sup> , b	12.9 ± 4.8	4.5 ± 3.4	1.3 ± 7.2
	7	36.7 ± 6.9	0.2 <sup>c</sup> ± 1.8	6.8 ± 4.9
	Avg.	31.9 ± 5.8	1.0 ± 2.2	5.4 ± 6.0
Average	4	38.1 ± 7.6	28.8 ± 9.8	22.9 ± 11.6
	7	49.3 ± 7.6	28.4 ± 9.0	29.1 ± 8.8
	Avg.	43.6 ± 7.6	28.6 ± 9.4	26.0 ± 10.2

\*This series was run earlier in the season than other series of the same vegetables.

<sup>b</sup>Only three runs; average weighted accordingly.

<sup>c</sup>Gain.

Although there were variations in the data on the same vegetables and on different vegetables, there was a consistently greater destruction of ascorbic acid in the vegetables stored in the A refrigerators than in vegetables stored in the same position in the H refrigerators or in vegetables stored in the vegetable container on the floor of the H refrigerators. Concurrent with the marked reduction in evaporation from the vegetables stored in the vegetable container in the H refrigerators, there was a tendency for the destruction of ascorbic acid to be less rapid during the first four days and more rapid during the next three

days than when these vegetables were stored outside the container but in the same refrigerators. At the end of seven days, however, the destruction was substantially the same.

The data in Table I are presented in condensed form in Table II and Fig. 1. In Table II the data are treated by the use of index numbers to make the comparison clearer. The index numbers indicate that the destruction of ascorbic acid averaged over 64 per cent more rapid in refrigerators A than in the more humid refrigerators II. The least difference was shown by spinach, which lost ascorbic acid 27 per cent more rapidly in refrigerators A. Supplementary experiments indicated that destruction of ascorbic acid in closed vegetable containers on the floor of refrigerators A also proceeded at a rate substantially more rapid than in refrigerators II, either in the open or in similar containers.

TABLE II

SUMMARY OF AVERAGE ASCORBIC ACID LOSSES IN GREEN VEGETABLES UNDER DIFFERENT STORAGE CONDITIONS

POSITION	PERCENTAGE LOSS			INDEX NUMBERS		
	REFRIGERATORS A	REFRIGERATORS II	REFRIGERATORS II (VEG. CONTAINERS)	REFRIGERATORS A	REFRIGERATORS II	REFRIGERATORS II (VEG. CONTAINERS)
AVERAGE HUMIDITY	65%	93%	98%	65%	93%	98%
Lettuce	43.2	29.9	23.5	144	100	78.5
Spinach	79.9	63.1	64.4	127	100	108
Parsley	24.6	14.5	9.6	170	100	66
Snap beans	40.4	28.2	32.0	143	100	113.5
Shelled limas	43.1	23.6	17.3	183	100	73.5
Shelled peas	31.9	1.0	5.4	3,190	100	540
Averages	43.85	26.7	25.4			
Index No.	164.3	100.0	95.1			
Index No.	172.6	104.9	100.0			

This extensive series of determinations indicates the hitherto unemphasized importance of the effect of evaporation on the ascorbic acid content of fresh green vegetables and demonstrates the importance of maintaining such foods in an atmosphere of high humidity and low air movement, as well as at a low temperature, to preserve their ascorbic acid content.

#### ASCORBIC ACID, BIO-ASSAY

The more extensive destruction of ascorbic acid in green vegetables stored in refrigerators A than in refrigerators II was confirmed by bio-assay<sup>3</sup> on two sets of young scorbutic guinea pigs.

Each day during a period of four weeks an equivalent of 0.7 Gm. (fresh weight) of parsley, which had been stored respectively in refrigerators A and II for six days, was fed to each animal as a supplement to the scurvy-producing diet. At the end of the test period the animals fed the extensively dehydrated parsley stored in the A refrigerators had lost 1.9 per cent in weight, while those fed parsley stored in the H refrigerators had gained 4.8 per cent in weight. The experiments from which these results were derived, however, were not sufficiently extensive to be more than corroborative of the chemical findings.

## VITAMIN A, PHOTOMETRIC

The amount of carotene (provitamin A) destruction was estimated, using a recording spectrophotometer<sup>4</sup> which plotted the reflectance curves of vegetables before and after storage in the A and H refrigerators and in the vegetable containers in the H refrigerators. These spectra were compared with those of alpha and beta carotene and oxidized carotene.

The results indicated that there was more destruction of the carotene in lettuce and carrots stored in the A refrigerators than in the more humid H refrigerators. No difference in carotene destruction was noted with respect to spinach, tomatoes, and Swiss chard.

Interpretation of results was made difficult because the spectra for the carotenes and the chlorophylls overlap in the regions of greatest absorption. An instrument which would read in the region of the ultraviolet maximum for carotene is needed.

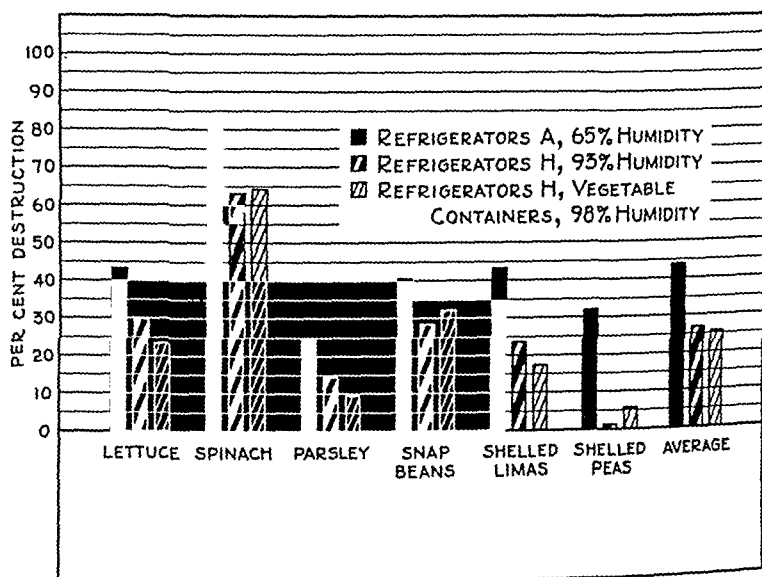


Fig. 1.—Average destruction of ascorbic acid in refrigerated green vegetables as influenced by evaporation.

VITAMIN A AND B<sub>1</sub> BIO-ASSAYS

Biological tests were performed to determine qualitatively whether vitamins A and B<sub>1</sub> like ascorbic acid show a tendency to be destroyed more rapidly at lower humidities. One group of rats made deficient in vitamin A<sup>5</sup> was fed a daily supplement of 60 mg. of lettuce (fresh weight) that had been stored for four days in the A refrigerators. A similar group was fed the same amount of lettuce that had been stored for four days in the H refrigerators.

One group of rats made deficient in vitamin B<sub>1</sub><sup>6</sup> was fed daily 1.00 Gm. of lettuce that had been stored for four days in the A refrigerators, while a similar second group was fed daily 1.00 Gm. of lettuce stored for the same period of time in the H refrigerators. The lettuce was comminuted and mixed into the diet.

In both instances the animals fed the lettuce stored in the H refrigerators showed better weight increases, indicating that the vitamin A and vitamin B<sub>1</sub> of lettuce is better preserved when vegetables are so maintained in refrigerated storage that evaporation is minimized.

#### SUMMARY AND DISCUSSION

An extended series of determinations indicated that the maintenance of vitamin C (ascorbic acid) in fresh green vegetables is considerably improved by storage at a high humidity and low air movement. During storage in domestic refrigerators the destruction of this vitamin proceeded at a rate averaging some 64 per cent greater for six green vegetables stored at an average humidity slightly below 65 per cent and with considerable air movement than when stored at the same average temperature (41° F) but at an average humidity of about 93 per cent and with low air movement. Biological and photometric measurements indicated that vitamin A and vitamin B<sub>1</sub> (thiamin) as well as vitamin C (ascorbic acid) were better preserved when vegetables were stored in more humid atmospheres.

Supplementary studies showed that the storage of fresh vegetables at high humidity and low air movement markedly retarded wilting and consequent wilting plasmolysis. It is customary for wilting plasmolysis to accelerate the oxidation of the cell constituents and impair the natural resistance of the plant to microbial invasion. This may explain why vegetables maintained in a humid and quiet atmosphere show better maintenance of vitamins and overall freshness.

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## FURTHER STUDY OF THE VI ANTIBODY CONTENT OF THE SERA OF TYPHOID PATIENTS AND CARRIERS

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THE development of Vi antibody to a titratable degree in the sera of typhoid patients has been shown to be sporadic and unpredictable (Almon and Stovall;<sup>1</sup> Almon, Read, and Stovall;<sup>2</sup> Felix, Krikorian, and Reitler<sup>3</sup>). Its occurrence in the sera of chronic carriers, on the other hand, seems to be a much more common occurrence. Felix<sup>4</sup> reports that the proportion of carriers in whose sera the antibody may be demonstrated is high enough to make it feasible to use this characteristic in the location of carrier suspects. Bhatnagar, Speechly and Singh,<sup>5</sup> and Pijper and Crocker<sup>6</sup> report that all carriers who are shedding organisms may be shown to have the Vi antibody. Although the results reported in this paper do not differ materially from those of other workers, the additional number of sera studied may add to the statistical value of the figures.

We have tried to choose a method both sensitive and accurate. The earlier work on the titration of the Vi antibody was done by absorbing the sera of their O and H antibody content and testing for the remaining Vi with a culture known to be highly agglutinable by this antibody. More recently, three direct methods, somewhat similar in principle, have been proposed for demonstrating this antibody. One of them, that of Detre,<sup>7</sup> consists essentially of the selection, by alternating agglutination and plating procedures, of a culture of *Eberthella typhosa* completely inagglutinable in typhoid O antiserum; and of the removal of the flagella from a suspension of this culture by repeated washings in saline. The resulting suspension is not agglutinated by O or H antibodies, but fully retains its sensitivity to the action of the Vi antibody. Bhatnagar, Speechly, and Singh<sup>5</sup> found what they described as a Vi variant, i.e., a culture which possesses no H antigen and shows little O and remains stable in this condition. They used it for the direct titration of the Vi antibody and found frequent control and rejuvenation procedures unnecessary. Their method, therefore, is like that of Detre except that their culture is stable in the Vi condition. The third method, described in detail by Felix,<sup>7</sup> makes use of carefully selected strains of organisms, but dispenses with removing the flagella, and standardizes their Vi agglutinability with a "provisional standard serum." It is this method which has been used by Felix for the titration of carriers' sera.

Since the work reported in this paper was begun before the details of Felix's method had been published and before the existence of Bhatnagar's culture had been announced, the method of Detre,<sup>5</sup> slightly modified, was the one employed. The cultures to be used were selected, as in the original method, by plating from the supernatant liquid of an agglutination tube in which those organisms

TABLE I

A COMPARISON OF THE RESULTS OF TITRATING SERA FOR VI ANTIBODY FOLLOWING ABSORPTION AND BY THE METHOD OF DETRE

CASE NO.	TIME SINCE ONSET OF ILLNESS	TITER BY DETRE METHOD	TITER FOLLOW- ING ABSORPTION
<i>Severe cases</i>			
9	44 days	0	0
11	18 days	0	0
20	{ 49 days	1:40	0
	{ 95 days	0	0
28	6 months	0	0
55	8 months	0	0
<i>Mild to moderate cases</i>			
1	{ 22 days	0	0
	{ 37 days	0	0
	{ 59 days	0	0
2	{ 4 days	0	0
	{ 41 days	0	0
3	21 days	0	0
5	45 days	0	0
6	{ 8 days	0	0
	{ 17 days	0	0
	{ 28 days	0	0
18	{ 42 days	0	0
	{ 50 days	0	0
19	68 days	1:40	1:40
30	43 days	0	0
40	{ 15 days	0	0
	{ 21 days	0	0
	{ 27 days	0	0
43	27 days	1:80	0
47	6 days	0	0
<i>Carriers</i>			
17	{ Not known	1:160	0
	{ Not known	1:160	0
22	28 years	1:40	0
23	35 years	1:40	0
31	No history of typhoid	0	0
37	4 years	1:40	0
38	20 years	1:40	0
39	No history of typhoid	0	0
42	17 years	1:80	1:80

sensitive to the O antibody had agglutinated and settled out. The organisms remaining in the supernatant liquid were, therefore, chiefly of the O resistant type. From the plates a number of colonies were transferred to slants and tested for their O agglutinability. These procedures were repeated until several strains completely inagglutinable in potent O antiserum were found. The cultures thus selected were seeded on plain agar plates, and after twenty-four hours the resulting growth was washed off with saline. The suspensions thus obtained were centrifuged and the sediment was resuspended in fresh saline, this being done six times. The final suspensions were tested for their O, H, and Vi agglutinability with highly potent antisera maintained for this purpose. Those suspensions which were totally inagglutinable in 1:40 and 1:80 dilutions of the O and H sera and were strongly agglutinated by the Vi serum were saved for subsequent tests. (This method of testing for the complete removal of the H antigen has been entirely satisfactory, and seems simpler than the India ink method included in Detre's original description.) Such suspensions, if not too highly diluted, maintained their Vi character for periods of at least two weeks in the refrigerator.

TABLE II  
VI, O, AND H ANTIBODIES IN PATIENTS' AND CARRIERS' SERA

CASE NO.	TIME SINCE ONSET OF ILLNESS	VI TITER	O TITER	H TITER
Severe cases				
59	{28 days	1:40	1:5,120	1:5,120
	{38 days	0	1:2,560	1:160
61	{9 days	0	1:320	0
	{21 days	1:80	1:80	0
(Fatal) 65	22 days	1:40	1:1,280	1:160
(Fatal) 66	16 days	0	1:640	1:2,560
Mild to moderate cases				
35	{24 days	0	1:640	1:640
	{28 days	0	1:640	1:640
53	?	0	1:160	1:80
54	?	1:160	1:5,120	1:2,560
56	{7 days	0	1:160	0
	{28 days	0	1:160	1:160
57	{10 days	0	1:1,280	1:160
	{28 days	0	1:640	1:160
58	{20 days	0	1:320	1:320
	{35 days	0	1:320	1:160
60	{10 days	1:40	1:1,280	1:1,280
	{18 days	1:40	1:640	1:640
62	{15 days	0	1:40	0
	{25 days	0	1:160	1:40
63	{28 days	0	1:640	0
	{31 days	0	1:640	0
64	14 days	0	1:160	0
Carriers				
44	10 years	0	1:160	1:80
67	6 years	1:40	1:1,280	1:40
68	29 years	1:80	1:5,120	1:160
69	No history of typhoid	1:80	1:2,560	1:1,280
70	4 years	1:80	1:160	1:640
71	No history of typhoid	1:40	1:640	1:160
76	No history of typhoid	1:80	1:640	1:160
77	3 years	1:80	1:1,280	1:160
78	28 years	1:320	1:640	1:5,120
79	8 years	1:80	1:320	1:640
80	2 years	1:80	1:640	1:160
81	32 years	1:80	1:640	1:640
82	40 years	0	1:80	1:160
83	37 years	1:160	1:80	1:160
26	2 years	1:160	1:80	1:320

The authors are indebted to Dr. E. S. Sanderson, of the University of Georgia, for collecting and sending the sera and histories of 11 cases reported in this table, and to Dr. Forsbeck, of the Michigan Department of Health, for similarly furnishing us with 13 sera from carriers.

The experiments here reported deal with (a) the retitration by the Detre method of what remained of the sera reported upon in a previous paper (Almon, Read, and Stovall<sup>2</sup>); (b) the titration of sera from 14 patients more recently made available; (c) the titration of sera from 15 carriers also just recently made available.

A comparison of the titers of the Vi antibody, as determined by the absorption method and by the method of Detre, is presented in Table I. The inagglutinability of the Vi suspension in O and H antisera was controlled in every series of agglutination tests by setting it up in 1:40 and 1:80 dilutions of rabbit antisera having high O and H titers. The figures show that the presence of Vi antibody, as demonstrated by the classical means, was confirmed in each case by the method of Detre. This was true also in some tests on rabbit antisera, not included in the table. Not only was the presence of the

Vi antibody confirmed by the new method in those sera which had been shown by the old method to contain it, but 6 human sera and 5 of a total of 31 rabbit sera tested, which had given negative results by the absorption method, were shown by the more direct method to contain the antibody, thus giving evidence for the greater sensitivity of the direct method. In the light of these results the newer method was the only one employed in the subsequent work.

The results obtained by the titration of the sera recently acquired from cases and carriers are given in Table II. In order to make this report parallel with previous ones, the titers for O and H antibodies are also included.

From Tables I and II in this paper and from Table II in the paper published previously (Almon, Read, and Stovall<sup>2</sup>) the essential findings concerning the Vi antibody content of human sera are brought together in summary form in Table III. These data substantiate our previous conclusion that the appearance of the Vi antibody is not demonstrably correlated with severity of symptoms of typhoid infection nor with any particular stage of active infection. The correlation with the existence of the carrier state seems to be more definite. It is possible that even a greater percentage of positive results among carriers' sera might have been obtained if all of these originally studied had been available for retitration by the Detre method. In the original study only two of eleven carriers were shown to have the antibody in their blood, but upon retitration of those samples (seven in number) which had not been used up, all but two were shown to contain the Vi antibody. Of those which were not available for restudy, one had been shown by the absorption method to contain Vi antibody. This leaves two still in question. But at best our figures for the occurrence of the antibody among carriers would be somewhat lower than those of Felix,<sup>6</sup> of Bhatnagar and his co-workers,<sup>3</sup> and of Pijper and Crocker.<sup>9</sup>

TABLE III  
SUMMARY OF PRESENT AND PREVIOUS WORK CONCERNING THE OCCURRENCE OF THE VI ANTIBODY IN HUMAN SERA

	NO. OF PATIENTS INVESTIGATED	NO. POSITIVE FOR VI	PER CENT POSITIVE
Severe cases	14	6	43
Mild to moderate cases	26	9	34
Carriers	26	20	77

It is conceivable that with more refined methods, tissue reactivity to the Vi antigen may be detected in still more individuals who have been infected with the organisms, since nearly all of the evidence points to the occurrence of this antigen in most freshly isolated cultures from patients and carriers alike (Felix, Krikorian, and Reitler;<sup>8</sup> Craigie and Brandon;<sup>4</sup> Welch and Mickle;<sup>10</sup> Almon, Read, and Stovall<sup>2</sup>). This detection might be accomplished by the use of lower dilutions of the sera if complications due to factors other than the Vi antibody could be ruled out. Felix and his co-workers in all of their work dealing with this antibody have reported titers as low as 1:5, but we have never been able to satisfy ourselves in our own work that results with such low dilutions were reliable. However, the procedure of measuring cir-



culating antibodies is at best a crude one for the study of immunity. The fixed tissue antibodies in this disease remain entirely uninvestigated.

#### SUMMARY

By a direct titration method 15 of a total of 40 typhoid patients investigated and 20 out of 26 typhoid carriers were found to have demonstrable amounts of Vi agglutinin in their blood. It is concluded that the correlation between the presence of this antibody and the existence of the carrier state is significantly high, but no attempt is made at interpretation of this phenomenon.

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### CACHEXIA RESPONDING TO EXTRACT OF THE ANTERIOR LOBE OF THE PITUITARY\*

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SIMMONDS<sup>1</sup> described a cachexia due to destructive lesions of the anterior lobe of the pituitary gland. He originally described the syndrome as one of premature aging, atrophy of the genital organs with loss of sexual characteristics, and loss of hair. He believed the etiology to be that of a complete destruction of the pituitary gland by embolism. Later, however, patients were described in whom there were destructive lesions of the pituitary gland due to neoplasm or aneurysm, and in many no cause has been found.

Lisser and Escamilla<sup>2</sup> analyzed the symptoms of 134 clinical cases of Simmonds' disease, of which 69 were verified. The symptoms found in the verified cases reveal that females predominate over males 2 to 1. There was marked loss of weight, with an average weight of 91 pounds; in fact, weight loss is

\*From the Department of Medicine, Northwestern University Medical School and the Robert Bruce Preble Research Laboratory, Cook County Hospital.  
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suggested as a cardinal symptom. Asthenia was present in 83 per cent; psychic apathy and somnolence were present in 48 per cent; anorexia in 33 per cent; and vomiting in 30 per cent. Diabetes insipidus was described in 13 per cent; sensitivity to cold was present in 41 per cent; amenorrhea in 52 per cent. Libido was lost by 58 per cent of the males. Premature aging occurred in 46 per cent and dry skin in 56 per cent. Loss of axillary and pubic hair was observed in 38 per cent. The basal metabolic rate averaged  $-31$ , and a low body temperature was found in 78 per cent.

A careful review of the literature fails to reveal case reports that can be closely correlated with the following report of a man who has been under observation since December 26, 1937, with the possible exception of a case report by Osgood.<sup>3</sup>

Osgood observed a female, who, although she had a voracious appetite, persistently lost weight, and became greatly emaciated. She had an achlorhydria, low basal metabolic rate, and a persistent diarrhea. She was treated for a time with antuitrin-S. Four years later she died from pulmonary tuberculosis, but at autopsy the pituitary gland was found to be normal. Osgood reports this case as an instance of questionable pituitary cachexia.

L. W., a 64-year-old white laborer, entered Cook County Hospital December 26, 1937. His immediate complaints were diarrhea, loss of weight, and edema of the feet, which had come on suddenly three weeks previously. He was irritable and after a short stay left the hospital. He was readmitted on January 27, 1938, with the same complaints. He stated an operation had been performed in 1915 for a gastric ulcer (according to his history, a part of the stomach had been removed and a new opening made). However, repeated x-rays of the stomach and direct gastroscopy gave no evidence of a gastroenterotomy. Since the time of his operation there had been no recurrence of his gastrointestinal symptoms. The edema of the feet had been noticed at various times during the past three years; in fact, during that period he had been treated at a dispensary as a chronic cardiac patient. The remainder of the history was essentially negative.

At the time of examination he was pale, emaciated, weak but not acutely ill. His temperature was  $98.2^{\circ}$  F.; pulse rate 48, respiratory rate 9, and blood pressure 126/80. He showed evidence of marked weight loss. An examination of the chest and abdomen revealed no abnormalities. Reflexes were normal. A slight edema of the ankles was present, which, however, disappeared with bed rest.

He became progressively weaker, with a persistent diarrhea of from 5 to 12 stools a day. These stools were yellow, watery, and of low specific gravity, but contained no blood. There was a complete achlorhydria after an ordinary Ewald test meal. Repeated x-rays of the gastrointestinal tract failed to reveal any abnormality. Direct gastroscopic examination by Dr. Schindler revealed a few superficial erosions upon a reddened mucosa. A proctoscopic examination showed a reddened mucosa. The basal metabolic rate was  $-34$ . Blood and spinal Wassermann tests were negative. There were no abnormal constituents in the urine. Blood chemistry showed nonprotein nitrogen 25, urea 15.41, uric acid 2.5, cholesterol 160, sugar 86, total protein 6.83, albumin 4.37, globulin 2.46, and ratio 1.77.

Shortly after entering the hospital a severe glossitis developed. Neither the glossitis nor the diarrhea was improved by the administration of nicotinic acid or intramuscular liver extract.

During his entire stay in the hospital his appetite was better than average, and, although he was given a high caloric diet, the weight loss continued. The basal metabolic rate remained low, averaging from  $-26$  to  $-34$ . From a normal weight of 180 pounds his weight gradually decreased to 96 pounds. By this time he had become so weak that he was hardly able to lift his hands.

Various forms of therapy were tried during the period up to October, 1938. During this time he was treated with high caloric and high vitamin diets, intramuscular injections of liver extract, and, at various times, thyroid, insulin, and nicotinic acid.

During this period there had been considerable discussion as to the cause of his illness, and it was finally decided that although he did not present a typical picture of a Simmonds' disease patient, he should be treated for the disease. Accordingly, beginning October 19, 1938, he was given daily 1 c.c. of extract of the anterior lobe of the pituitary gland\* intramuscularly, contained no water-soluble vitamins. By April 8, 1939, he had gained 70 pounds, his weight being 161 pounds. Shortly after beginning administration of the extract of anterior lobe of pituitary the patient began to feel better. His diarrhea decreased, and after thirty days he began to gain weight and strength. By May, 1939, he weighed 167 pounds and his basal metabolic rate was -10.



A.

B.

Fig. 1.—A, December, 1938. Weight about 100 pounds. B, May, 1939. Weight about 160 pounds.

The pituitary extract was discontinued in May, 1939. The patient continued well until October 15, 1939. After a loss of 9 pounds and a recurrence of the diarrhea, he was given 1 c.c. of the gonadotropic hormone of the pituitary gland daily for thirty days, with prompt relief of the diarrhea and return to his previous weight level. He is now up and about the ward, although he has not fully regained his strength. Otherwise he appears to be perfectly normal.

The gonadotropic hormone was not continued because of the belief that it was impossible to procure an extract free of the other hormones.

The history of a patient seen in private practice seems to be a partial parallel of the preceding case.

\*The extract of the anterior lobe of the pituitary gland, "polyanson," of Armour & Company was used throughout. The method of preparation of polyanson should destroy all water-soluble vitamins.

Mrs. T. R., was seen on August 14, 1939. She had recently moved to Evanston from Colorado. Her initial complaint was that of weakness, loss of weight, and emaciation. She stated that during the past five years she had continuous diarrhea of from three to numerous liquid stools daily. This diarrhea had been present at various times for the past fifteen years. Menses were irregular, the quantity greatly decreased and usually accompanied by pain.

An examination revealed an emaciated white woman, 44 years of age. Her facies were striking; she was wrinkled and looked many years older than the age given. She had the peculiar wizened expression seen in the hypopituitary dwarf and a slight glossitis. Her weight was 92 pounds.

Examination of her blood showed hemoglobin 14.5, red blood cells 4,800,000, white blood cells 6,050, total nonprotein nitrogen 29.5, glucose 91, cholesterol 213, and chlorides 485. Basal metabolic rate was -7. Gastric analysis revealed free hydrochloric acid 6; total acidity 22. X-ray of the gastrointestinal tract was entirely negative.

Repeated examinations of the stools revealed a trace of blood on one occasion. Amoebae were not found although they had been reported on one previous examination.

This patient varied from the preceding one in that her appetite was poor. At one time she had gained considerable weight while on forced feedings in a sanatorium under the care of a neurologist. Because of the peculiar facies, she was given the same extract of the anterior lobe of the pituitary as the first patient; slight glossitis, which was present, immediately improved. By February, 1940, she had gained 13 pounds in weight, her strength was sufficient to take care of a household, a four-room apartment, and normal social activities. She still has some diarrhea, especially when she is under a nervous strain. The menstrual periods have become more regular, normal, and painless.

#### DISCUSSION

With the exception of the loss of hair the patient described as L. W. answers well the description of Simmonds' disease. He had extreme asthenia, was apathetic, had lost his libido, and had lost weight persistently. He had the low basal metabolic rate described in pituitary cachexia, and because of this a myxedema was considered. He showed no reaction to thyroid therapy. He was given insulin in the hopes of increasing a utilization of sugar. With a diagnosis of an avitaminosis he was given for a long period a high caloric diet with vitamins added in the form of yeast and intramuscular injections of a liver extract. Because of the acute glossitis he was given sufficient quantities of nicotinic acid to cause amelioration of such symptoms. It is interesting to note that the glossitis did not disappear until he had been given the extract of the anterior lobe of the pituitary gland.

In the second patient, the sole criteria for diagnosis have been the peculiar facial expression, the loss of weight, the extreme asthenia, and the unstable nervous system. It is true in her case that anorexia nervosa must be considered. Anorexia nervosa is the most common condition confused with Simmonds' disease. In this instance the diarrhea is a symptom not usually present in anorexia nervosa.

She had received considerable care during the preceding five years, when she had been treated by one physician for an amoebic dysentery and by others for a neurosis.

Within two weeks after the institution of the intramuscular injections of extract of the anterior lobe of the pituitary, there were a marked change in her emotional stability and a definite gain in weight and strength. This improvement has continued up to the present time, with a total gain of 13 pounds.

It is true that she still has spells of diarrhea, which consist of 3 to 4 stools, usually in the morning. The total quantity of feces expelled by such diarrheal attacks is considerably less than a pint. These attacks occur as the result of situations that upset her mentally, and are especially likely to occur just before attendance at some social function.

#### CONCLUSIONS

1. Two persons with cachexia are reported greatly improved or fully recovered following the administration of extract of the anterior lobe of the pituitary gland.

2. It is believed that these two patients represent atypical instances of Simmonds' disease or dysfunction of the anterior lobe of the pituitary.

3. It is possible that the second patient represents a case of anorexia nervosa, although the response to the glandular therapy makes it appear proper to include her in these case reports.

4. The prompt recovery of the glossitis in both patients, and of the red-denied rectal mucosa in the first patient, obviously suggests a relationship between the function of the anterior lobe of the pituitary gland and the utilization of the vitamin B complex. Evidence in support of this suggestion will be presented in a forthcoming paper on pellagra.

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30 NORTH MICHIGAN AVENUE

# LABORATORY METHODS

## AN APPARATUS FOR CONSTANT INTRAVASCULAR INJECTION OF LIQUIDS\*

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THE apparatus here described is characterized by its simplicity of construction and its high degree of efficiency. It was designed for use in experimental investigations in which it is desired to introduce various liquids into the blood stream (vein or artery) at a constant rate of flow.

Repeated tests of the apparatus, performed by causing it to deliver water into microburettes, have demonstrated that both the rate of flow and the volume of water delivered per unit of time remain constant during observations conducted over a period of one to one and one-half hours, i.e., the period required to completely empty the syringes used when filled to capacity.

The essential construction of the device includes a constant speed motor which drives a nuggled disk by a hard rubber friction drive wheel that is attached to the revolving shaft of the motor. The disk operates a pinion and gear reduction to a long screw which is thus kept in a constant forward motion and propels the piston of one or more syringes. An adjustment is provided on the screw to automatically stop the motor when the pistons have reached the end of the barrel of the syringe.

The only adjustment or replacement that may be required, after long continued use of the apparatus, is that due to slight wearing down of the circumference of the rubber friction drive wheel. When necessary the wheel can be replaced with a new one in a few minutes. It is advisable also to clean the nuggled surface of the disk occasionally to prevent accumulation of dirt which may sometimes cause the drive wheel to slip in spots.

A synchronous motor is essential for constant speed. Such a motor, however, has much less power than a shaded pole induction motor of the same size. In most constant injection experiments, a physiologic range of constancy can be obtained with the induction motor. The motor variation under a continuous load is insignificant, sometimes not more than about 0.2 per cent. We have found the shaded pole induction motor† entirely satisfactory in our experimental work involving constant injections of aqueous or physiologic saline

\*From the Laboratory of Experimental Endocrinology, School of Medicine, University of Pittsburgh.

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†Obtained from the Merkle-Korff Gear Co., Chicago, Ill.

solutions. The apparatus is illustrated in Fig. 1. The following is a detailed description of the apparatus, its construction and operation.

The motor (1) is set on two rods (2) so that its position can be adjusted in relation to the nuggled disk (7) by manipulating the handle that is attached to a screw (3) which moves the motor forward or backward on the supporting rods. A line or arrow on the side of the motor serves as an indicator that can be adjusted, by moving the motor, to various positions on a scale (4), placed alongside and just below the motor. This enables the observer to determine the speed of propulsion of the piston (17) in the syringe (18) and the corresponding rate of delivery of its contents after a preliminary determination is made for the syringe employed. Speed of propulsion increases as the drive wheel is moved from circumference toward the center of the nuggled disk, and vice versa.

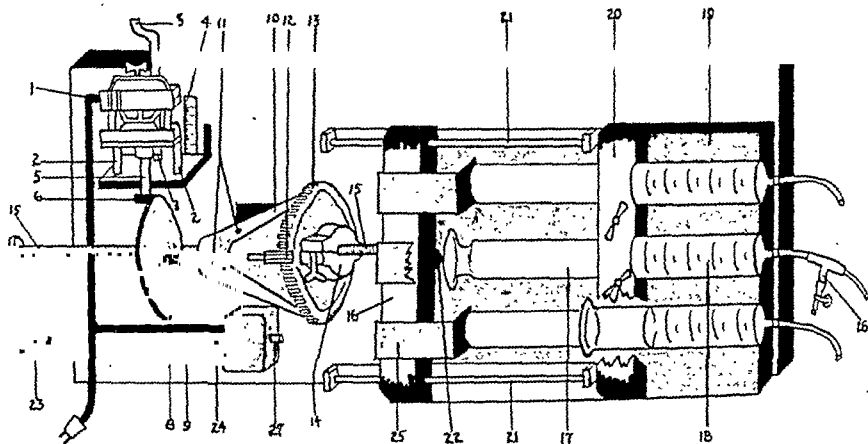


Fig. 1.

The motor rotates a shaft (5) at 50 r.p.m. At the end of the shaft is attached a hard rubber friction drive wheel (6), 2.5 cm. in diameter and 4 mm. wide at the circumference, which rests against a nuggled disk (7). The disk, 9 cm. in diameter, is held firmly in contact with the drive wheel by the aid of a spring (8) which is coiled around a portion of the drive shaft (9) that protrudes from the support (10). When the motor is moved forward or backward, pressure on the disk toward the support permits the drive wheel to be adjusted in the desired position without friction against the disk; releasing the disk causes it again to contact firmly with the drive wheel. Lubrication of the drive shaft is provided for by oil holes (11) in the support.

The shaft operates a pinion (12) which drives a geared wheel (13) to which is attached a split clamp (14). Within this clamp are the threads (see Fig. 2) that propel a long (30 cm.) threaded rod or screw (15) as the clamp rotates with the wheel to which it is fastened. The long screw passes through the support and the geared wheel without contact, and it is propelled forward without rotating.

At the forward end the screw is fastened to a wooden block (16) which propels the piston (17) of one or more syringes (18) that lie in grooves of a wooden support (19) where they are held firmly by a grooved wooden clamp (20) that constitutes the upper part of the support. The block is moved along on two lubricated rod supports (21) and to it are fastened rubber knobs (22) which press against the heads of the pistons, propelling them in the cylinders of the syringes.

A set screw (23) is adjusted to the outer end of the long screw so that it contacts the "off" button on the switch (24), when the syringe is empty, and automatically stops the motor.

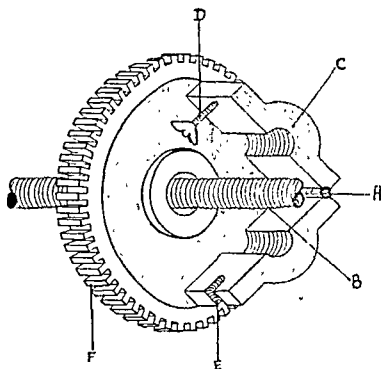


Fig. 2.

Fig. 2 illustrates the construction and operation of the split clamp (14) which propels the long screw (15) that controls the movement of the piston in the syringe. If it is desired to continue an injection beyond the period required to empty the syringe (or syringes), refilling the syringe requires only a few seconds. The split clamp is opened by loosening the nut on the threaded bolt (D) which fits into a groove (E) on the opposite half of the clamp. This permits the clamp to open at the hinge (A), releasing the threads (B) of the long propelling screw (15, Fig. 1) which is driven by the threads (C) in the split clamp as it revolves with the geared wheel (F) to which it is attached.

To refill the syringes, the block which propels the pistons of the syringes is pushed back so that the screw passes unrestricted through the split clamp, geared wheel, and support, to the original starting position. A metal saddle (25), which rides on top of the block, is notched so that it grasps the head of the piston of the syringe. As the block is displaced backwards the saddle draws back the piston and the fluid to be injected is drawn into the cylinder of the syringe through a side tube (26) provided for this purpose. While this is done, the clamp is removed from the rubber tubing on the glass side tube (26, Fig. 1) and



placed on the tubing attached to the syringe. The clamp is replaced on the side tube before starting the motor again for continuing the injection. The split clamp is again closed around the propelling screw, and the motor started by pressing the "on" button (27) of the switch.

The apparatus at present employed in this laboratory is provided with three 50 c.c. syringes. Using one syringe, with the drive wheel adjusted at the outer margin of the nuggled disk, it delivers 0.7 c.c. per minute; if the drive wheel is moved toward the center of the disk, the speed is increased until 2.5 c.c. per minute is delivered when the drive wheel is close to the center of the disk. Thus the range is from 0.7 c.c. (one syringe) to 7.5 c.c. (three syringes) per minute. Of course, it is possible to increase the number of syringes employed, permitting the performance of a number of experiments with a corresponding number of animals at the same time.

The speed of propulsion can be reduced by employing drive wheels of smaller diameters and by using disks of greater diameters. Furthermore, the quantity of liquid delivered can be reduced by employing syringes with cylinders of smaller diameters. Thus, by a combination of these factors, which can easily be introduced into the construction of the apparatus, it is possible, quantitatively, to vary greatly the speed and volume in the delivery of the fluid injected.

The author is indebted to Mr. Ben Supovitz for drawing the illustrations.

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## THE PHOTOELECTRIC MICRODETERMINATION OF NITROGENOUS CONSTITUENTS OF BLOOD AND URINE BY DIRECT NESSLERIZATION\*

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WHEN Nessler's solution is added to a dilute solution of ammonium salts, an amber-red color is produced which is proportional to the concentration of ammonia in the solution and which can, therefore, be used for the colorimetric estimation of the ammonia. However, if the concentration of ammonia is too great, the colored substance is precipitated. Even in dilute solutions precipitation or clouding occurs on standing; but before it occurs, the color becomes progressively more intense. Many factors other than time also influence the intensity of the color or the speed of cloud formation. Among these are the following: the proportion of Nessler's solution used to the final volume of the solution, the concentration of salts, such as sodium sulfate; the pH of the final solution; the presence of substances which form precipitates with mercury ions; traces of organic solvents, particularly acetone; substances other than ammonia which produce a color with Nessler's solution. To these factors should be added

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the constant presence in almost all laboratory reagents as well as on laboratory glassware, filter paper, and other equipment, of traces of ammonium salts or of substances producing ammonia on ashing, or of substances influencing the color produced by Nessler's solution.

Accurate determination of ammonia nitrogen by nesslerization must take all these factors into consideration. The technique employed must minimize these sources of error and make them constant both in the standard and in the unknown solutions. One of the methods of minimizing the interfering factors is to aerate or distill the ammonia formed into an acid solution and then to nesslerize this solution. However, if such procedures are used, the determinations are then more conveniently and accurately made by volumetric rather than by colorimetric methods. It is to avoid aeration or distillation, which is usually a macroprocedure and which is time-consuming and requires elaborate apparatus, that most biological laboratories make use of methods involving direct nesslerization. Many of these methods have not only the usual subjective errors of ordinary colorimetry but also the much more significant errors produced by failure to consider the many factors which influence the color produced by Nessler's solution.

The photoelectric colorimeter, when used with the proper light filter and with precautions of producing constant conditions, permits much more accurate as well as more rapid determinations than do instruments which require subjective color matching. Furthermore, since pale colors are read with as great accuracy as more intense ones, the photoelectric instruments make possible an ultramicrotechnique unthought of before in routine laboratories. However, the unmodified adaptation of the crude methods of ordinary colorimetry to the photoelectric colorimeter would hardly be worth while; for, though the subjective errors would be removed, the gross technical errors would be untouched. Besides this, other errors are introduced because of a fundamental difference in the two techniques. In color matching constancy of conditions is not so important since unknown and standard are made up simultaneously, whereas in photoelectric methods standard curves are made up at one time while the unknown may be analyzed months or years later with different or aged reagents, and at a possibly different temperature. The adaptation, then, of colorimetric methods to the photoelectric colorimeter requires careful study of all these sources of error. If, however, these sources are eliminated or controlled, the resulting methods are refined and rapid, and have the accuracy of the best volumetric methods.

The following procedures for the determination of the nitrogenous constituents of blood and urine by direct nesslerization were designed to offer such advantages. They are based on the micro-Kjeldahl technique of Koch and McMeekin.<sup>1</sup> The amber solutions obtained have absorption bands between 400 and 450  $m\mu$  and are, therefore, best analyzed with a blue filter (Cenco filter No. 1, which, with an incandescent source of light, has an effective maximum transmission at 430  $m\mu$ ). About 0.025 mg. of ammonia nitrogen is used for the final color formation, which, with 3 c.c. of Nessler's solution in a final volume of 20 c.c., gives readings around 50 on the microgalvanometer when water is set at 100. The amount of sulfuric acid chosen is the least possible for convenient ashing, so as to minimize the concentration of the sodium sulfate in the final solution.

In the determination of nonprotein nitrogen a trichloroacetic acid filtrate of blood has been chosen for analysis rather than the usual Folin-Wu filtrate, because the latter solution contains substances which when ashed may produce clouding on addition of Nessler's solution. It will be noticed that all standard curves are made by determination of standard solutions subjected to the same procedures as the unknown solutions, so as to include the blank in the curve. That the blank values are different for different procedures can be recognized from Fig. 1, where the readings for the same amount of nitrogen are different in the three procedures.

#### METHOD

*Reagents and Apparatus.*—1. 2.4 N  $\text{H}_2\text{SO}_4$ . Into a 1,000 c.c. beaker, containing about 600 c.c. of water, add slowly with stirring 67.2 c.c. of concentrated sulfuric acid. Cool and make up to a liter in a volumetric flask. (Keep this, as well as other reagents, protected from ammonia vapor.)

2. 1.2 N  $\text{H}_2\text{SO}_4$ .

3. 30 per cent  $\text{H}_2\text{O}_2$ , C.P.

4. 5 per cent trichloroacetic acid.

5. 22.5 per cent  $\text{Na}_2\text{SO}_4$ . This solution is made up from anhydrous sodium sulfate with water at 37° C. and kept in an incubator at that temperature.

6. Nessler's solutions (Koch's modification<sup>1</sup>). Several liters should be made up at a time and kept in a tall bottle. A small amount of precipitate settles to the bottom on standing. It is futile to filter this off, as it will form again. The quantity needed for the day should be pipetted off from the top. The solution will keep indefinitely.

7. Pyrex 25 c.c. test tubes, marked for 20 c.c. with a file. These should be washed with distilled water just before using. Other test tubes, which must be dry at the time of use, should be kept stoppered when not in use.

*The Determination of Nonprotein Nitrogen.*—One-half cubic centimeter of blood or serum is mixed in a small dry test tube (15 by 125 mm.) with 2.0 c.c. of water and then treated with 2.5 c.c. of 5 per cent trichloroacetic acid. (When urine containing protein is to be similarly analyzed, it should first be diluted so that the nonprotein nitrogen concentration is not more than 100 mg. per 100 c.c. The diluted solution is then treated as previously.) After being thoroughly stirred and allowed to stand for several minutes, the mixture is filtered on a folded 7 cm. filter paper, which has been handled as little as possible. (Whatman No. 40 filter paper has been found satisfactory.) The first few drops of filtrate are caught in the original tube and refiltered. Exactly 0.5 c.c. of this filtrate is then transferred to a pyrex test tube marked for 20 c.c. To it is added 0.5 c.c. of 2.4 N sulfuric acid. The tube is placed in a bath of saturated calcium chloride and heated for several minutes at about 130° C. until charring begins. (This preliminary step may be omitted, but it serves to remove the water and chloroform, formed from the decomposition of the trichloroacetic acid, without the annoying spattering that occurs when the tube is heated immediately over a free flame.) The tube is then removed from the bath and heated with constant agitation over a free flame until fumes of sulfur trioxide fill the tube. After about forty-five seconds of cooling, 1 drop of 30 per cent hydrogen

peroxide is added from a capillary pipette directly to the sulfuric acid. Decolorization is immediate, but the tube is heated again with agitation for about two minutes, at which time the ashing is complete. The cooled solution is diluted to about 15 c.c. and treated with 3 c.c. of Nessler's solution from a pipette reserved for that purpose. It is then made up to 20 c.c., thoroughly mixed with the aid of clean rubber stopper, and read in the photoelectric colorimeter within two minutes.\* When several determinations are made at the same time, it is more accurate to make the reading after each nesslerization rather than to nesslerize all tubes at one time. However, readings at five minutes are usually not more than 0.3 lower than those at two minutes.

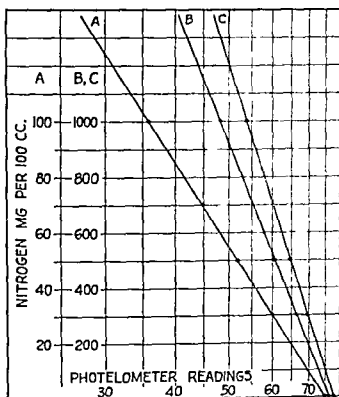


Fig. 1.—The relationship between photometer readings and concentration of nonprotein nitrogen (Curve A), concentration albumin nitrogen plus nonprotein nitrogen (Curve B), and total nitrogen (Curve C). In Curve A, 0.02 mg. nitrogen reads 55.5; in curve B, it reads 55.8; and in Curve C, it reads 58.0.

Calculation is made by reference to a curve or table of values obtained from standard solutions. For this purpose 4.7166 Gm. of purest ammonium sulfate are dissolved in 100 c.c. of water. This is the stock solution for all the nitrogen analyses described here. It contains 10 mg. nitrogen per cubic centimeter. If all the curves required are made within a day or two, it is unnecessary to add any preservative to this solution, which thus eliminates a possible source of error. Of this stock solution, 3, 5, and 7 c.c. are diluted to 100 c.c. in volumetric flasks, making solutions containing 30, 50, and 70 mg. nitrogen per 100 c.c., respectively. Analyses of these solutions, as well as of a blank of water alone, are carried out exactly as for blood, including the filtration and ashing. With the proper light filter it will be found that the points, when plotted on semi-logarithmic paper as in Fig. 1, fall on a straight line.

*The Determination of Total Nitrogen in Serum and Urine.*—To 20 c.c. of 2.4 N sulfuric acid in a test tube is added 0.1 c.c. of serum or urine measured in

\*The Cenco-Sheard-Sanford photometer,<sup>2</sup> manufactured by the Central Scientific Co., was used in all these experiments.

a pipette calibrated "to contain." The pipette is washed out several times with the sulfuric acid in the tube. Exactly 0.5 c.c. of the well-mixed solution is transferred to a pyrex test tube marked for 20 c.c. and heated for several minutes in a boiling water bath. (This preliminary step may be omitted, but it serves to hydrolyze the protein and thus prevents foaming.) The tube is then heated in a free flame with rapid agitation until charring occurs and sulfur trioxide vapors fill the tube. After forty-five seconds of cooling, a drop of 30 per cent hydrogen peroxide is added; this decolorizes the solution. The heating is then continued gently for two minutes. The tube is cooled under the tap, and water is added to about 15 c.c., followed by 3 c.c. of Nessler's solution. The solution is made up to 20 c.c., mixed thoroughly, and read in the colorimeter within two minutes. The complete determination can be made in less than ten minutes.

Calculation is made by reference to a table or curve of values obtained from standard solutions. The stock solution, containing 10 mg. nitrogen per cubic centimeter, is diluted with water to give solutions containing 5 and 3 mg. nitrogen per cubic centimeter. These solutions, as well as water alone and the stock solution, are analyzed as above by mixing 0.1 c.c. of each with 20 c.c. of 2.4 N sulfuric acid, using the same pipette calibrated "to contain." One-half cubic centimeter is ashed, treated with hydrogen peroxide, diluted, treated with 3 c.c. of Nessler's, and made up to 20 c.c. The points should fall on a straight line when plotted on semilogarithmic paper, even when extended up to 14 mg. nitrogen per cubic centimeter (see Fig. 1). The total protein concentration is calculated from the total nitrogen in the usual way by subtracting the non-protein nitrogen concentration and multiplying by 6.25. In the analysis of concentrated urines, if the reading obtained shows a total concentration higher than 14 mg. per c.c., it is better to dilute the urine with an equal quantity of water and repeat the determination.

*The Determination of Albumin and Globulin in Serum (After Horvath).*—To 3 c.c. of 22.5 per cent sodium sulfate in a small test tube kept in a beaker of water at 37° C., is added 0.1 c.c. of serum from a pipette calibrated "to contain." The pipette is washed out several times with the sodium sulfate solution. The contents of the tube are mixed by inversion, using a clean rubber stopper. The tube is replaced in the beaker of water which is now kept in an incubator at 37° C. for two hours. The precipitated globulin is now filtered off on a folded 7 cm. filter paper (Whatman No. 42). The first few drops of the filtrate are caught in the original tube and refiltered. The filtrate should be perfectly clear. If it is not, a finer grade of filter paper must be used. It is not necessary to filter off more than a few drops of liquid. Two-tenths cubic centimeter of this filtrate are transferred with a pipette calibrated "to contain" into a tube containing exactly 2 c.c. of 1.2 N sulfuric acid. The pipette is washed out several times with the sulfuric acid solution. Of this mixture exactly 1 c.c. is transferred to a pyrex test tube marked for 20 c.c. and ashed as in the previous procedures with the aid of a drop of 30 per cent hydrogen peroxide. It will be found that the final clear solution solidifies on cooling because of the considerable amount of sodium sulfate present. About 15 c.c. of water are added with stirring to dissolve these crystals. The well-mixed solution is treated with 3 c.c. of Nessler's reagent diluted to 20 c.c., mixed, and read in the colorimeter within two minutes.

Calculation is made by reference to a table or curve of values obtained from standard solutions. The stock solution containing 10 mg. nitrogen per cubic centimeter, and dilutions containing 5 and 3 mg. per cubic centimeter, and water alone are all analyzed in the same way as serum, using the same pipettes and filtering with the same type of filter paper. The points should fall on a straight line when plotted on semilogarithmic paper (see Fig. 1). The values obtained represent albumin nitrogen plus nonprotein nitrogen. The albumin concentration is obtained by subtracting the nonprotein nitrogen concentration and multiplying by 6.25. Subtracting the albumin concentration from the total protein concentration gives the globulin concentration.

## COMMENT

The methods outlined involve no new principles but are offered as a technique which permits rapid but accurate determinations with minute amounts of material. They serve, too, to call attention to remediable errors in previous methods of direct nesslerization by insisting on constancy of conditions of the determinations and by inclusion of the blank in the determination of the standard solutions.

The substitution of ordinary test tubes for the large cumbersome 60 c.c. micro-Kjeldahl tubes used in most laboratories should be welcomed. However, for those who insist on using these larger tubes and who believe that a slightly less microprocedure would be more accurate, it is recommended that the same proportions of reagents and fluids to be analyzed as outlined here be used. If the amount taken for the final ashing is 2.5 times the amount suggested here and if the ashed material is made up to 50 c.c. with 7.5 c.c. of Nessler's solution, curves similar to those in Fig. 1 will be obtained with probably less relative blank from the filter paper.

The accuracy of these micromethods is attested by comparison with analyses made on the same material by macro-Kjeldahl procedures with titration of the distilled ammonia (see Table I).

TABLE I  
ACCURACY OF DETERMINATION OF NITROGENOUS CONSTITUENTS  
The results are expressed in mg. per 100 c.c.

SAMPLE	N.P.N. METHOD		TOTAL NITROGEN METHOD		ALBUMIN NITROGEN METHOD	
	AUTHORS'	MACRO- KJELDAHL	AUTHORS'	MACRO- KJELDAHL	AUTHORS'	MACRO- KJELDAHL
Blood	32.0	31.4				
Blood	38.8	38.1				
Serum	27.0	26.5	1,040	1,010		
Serum	24.5	23.7	1,200	1,205	760	780
Serum	26.5	27.5	1,180	1,162	775	800
Serum	23.0	23.2	850	843	432	425
Urine			1,350	1,310		
Urine	750	738				

## SUMMARY

A technique is offered for the microdetermination of the nitrogenous constituents of blood and urine by direct nesslerization, using the photoelectric colorimeter. It is rapid, convenient, and accurate.

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## A MICROPHOTOELECTRIC METHOD FOR UREA DETERMINATION IN BLOOD AND URINE BY DIRECT NESSLERIZATION\*

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MOST modern methods for the determination of urea in blood and urine involve the conversion of urea into ammonium carbonate by aid of the enzyme urease. The latter is usually obtained as a crude extract of jack bean meal. The ammonium carbonate formed has been analyzed in the more precise procedures by measuring the extractable carbon dioxide from the acidified solution, as in the manometric method of Van Slyke,<sup>1</sup> or by aerating the ammonia from the alkalized solution into a standard acid solution and titrating the residual acid.<sup>2</sup>

Neither of these two methods is widely used in biological laboratories where a large number of routine determinations of urea must be made daily. The manometric method requires special apparatus and a high degree of technical proficiency. The aeration method also requires special apparatus and is time-consuming. In this method 3 c.c. of blood must be used for each determination. Besides, if the air current is not controlled, there may be large errors. For these reasons, many biological laboratories have resorted to methods involving direct nesslerization. A number of these methods have appeared, the first and simplest of which was probably that of Karr,<sup>3</sup> who added urease to a Folin-Wu filtrate and nesslerized without attempting to remove the urease. Gruskin<sup>4</sup> was apparently the first of a series of investigators to advocate the addition of urease directly to blood and serum, and the subsequent removal of both the blood proteins and urease by a standard deproteinization technique. The filtrate obtained supposedly gave clear amber solutions with Nessler's reagent, the color of which was proportional to the amount of urea in the fluid.

When we attempted to adapt a method of direct nesslerization to the use of the photoelectric colorimeter, we were immediately impressed with the lack of accuracy of most of the procedures. There were two obvious sources of error which could be ascertained easily with the photoelectric colorimeter. The first was the difficulty in obtaining absolutely clear solutions on the addition of Nessler's solution, even when the urease was removed with the blood proteins. Both the blood and the urease solutions contained soluble substances which produced clouding on addition of Nessler's solution and which were not easily

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removed by any of the standard deproteinization methods. The second source of error was the variable amount of color-producing substances in the filtrates other than ammonia that had been formed from urea. If this source of error were constant, it might not produce much difficulty, for its value could easily be determined with the photoelectric colorimeter. But it was not constant and it was often quite large, representing from 3 to 10 mg. of urea nitrogen per 100 c.c.

A number of methods of deproteinization were investigated in an effort to obtain a filtrate from the blood-urease mixture which would produce clear solutions and which would contain a minimum of nonammonia color-producing substances. Folin-Wu filtrates were found unsatisfactory, as were those made with trichloroacetic acid and with toluene sulfonic acid. All these caused clouding. The use of cadmium sulfate and sodium hydroxide, recently recommended by Fujita and Iwatake<sup>5</sup> for the determination of true glucose because it yielded filtrates singularly free of nonsugar reducing substances, also proved disappointing. Clouding was obtained both in filtrates made in the cold and in those after heating. The Somogyi<sup>6</sup> procedure, using zinc sulfate and sodium hydroxide suggested by Peters and Van Slyke,<sup>7</sup> furnished filtrates that gave clear solutions with Nessler's reagent, but the blank obtained was large and variable. Besides, the filtration was annoyingly slow. However, when the mixtures were heated in a boiling water bath for two minutes, the subsequent filtration was rapid and yielded a filtrate that remained clear on the addition of Nessler's reagent and produced only a small and relatively constant blank.

Since in all photoelectric procedures, curves of values from standard solutions must be obtained by a technique that is constantly reproducible, it was found necessary to use a standard urease solution which was freshly prepared for each set of determinations in order to have the same blank from the urease. Obviously, the most exact method is to weigh out accurately the required amount of dry jack bean extract for each urease solution. This tedious procedure could be avoided by using urease tablets of approximately constant weight. Tablets of urease made by the Arlington Chemical Co. were found to be uniform and firm.

The colorimetric determination of urea involves the consideration of other sources of error inherent in all nesslerization procedures. These have been discussed in another paper.<sup>8</sup> It is probably of value to emphasize that inasmuch as no ashing is required here which would destroy any traces of the organic solvents used in the cleansing of pipettes, particular care must be employed to avoid their presence. Acetone is particularly harmful. The slightest trace of acetone has been found to produce sufficient clouding to cause large errors in this micro-method. For this reason, we no longer use acetone in cleansing and drying pipettes, but have returned to the use of alcohol and C.P. ether. There is an appreciable blank from filter paper. It is worth while, therefore, to use an ashless filter paper of uniform quality, such as Whatman No. 40.

#### METHOD

*Reagents.*—1. Urease solution. A tablet of Arleo-urease is ground in a clean dry mortar with exactly 10 c.c. of 0.05 disodium acid phosphate. The turbid



solution is transferred to a small tube reserved for this purpose. The solution must be used within two or three hours.

2. Zinc sulfate, 10 per cent. A trace of precipitate may form on standing. It settles to the bottom and may be ignored.

3. Sodium hydroxide, 0.50 N.

4. Nessler's Solution (Koch-McMeekin<sup>o</sup>).

*Procedure.*—Into a small dry test tube (15 by 125 mm.) 0.2 c.c. of blood or serum or urine, diluted 25 times, is transferred with a Van Slyke-Neill pipette. Then 0.2 c.c. of urease solution is added. The tube is rotated to assure thorough mixing, then stoppered with a clean rubber stopper, and placed in a water bath at 45° C. for fifteen minutes. Four cubic centimeters of water are added, followed by 0.3 c.c. zinc sulfate reagent, and 0.3 c.c. sodium hydroxide, with agitation of the contents of the tube after the addition of each reagent. The tube is placed in a beaker containing an amount of boiling water just sufficient to reach the level of the contents of the tube. After two minutes in the boiling water bath (which should not appreciably reduce the volume of liquid since the upper part of the tube acts as a condenser), the tube is cooled to room temperature and the contents filtered through a folded 7 cm. filter paper (Whatman No. 40). The first few drops are caught in the original tube and refiltered. Exactly 2 c.c. of the filtrate are transferred to a 25 c.c. test tube marked for 20 c.c. with a file. About 15 c.c. water are added, then 2 c.c. of Nessler's solution from a pipette reserved for that purpose. The solution is made up to 20 c.c., mixed by inversion with the aid of a clean rubber stopper, and read in the photoelectric colorimeter\* within two minutes. A blue filter, with a maximum transmission at 430 m $\mu$  (Cenco No. 1), should be used. A blank determination can be made by treating the blood or diluted urine as in the determination of urea by adding the zinc sulfate and sodium hydroxide before the urease and heating immediately for two minutes without any previous digestion. For blood we have found the blank to be of the order of 2 mg. urea nitrogen per 100 c.c., which can be assumed for all determinations. However, the urine blank includes the value of the preformed ammonia and must, therefore, be determined in each case. This determination, too, might theoretically be avoided if a permutit-treated urine is used for the determination, but since even such liquids may still show appreciable and variable blanks, it is better to avoid the use of permutit and to determine the blank which includes the preformed ammonia.

Calculations are made by reference to a table or curve of values obtained by analyses of standard solution of urea. A stock solution of urea, containing 80 mg. urea nitrogen per 100 c.c., is made up by dissolving 1.7144 Gm. of pure, dry urea in water and making up to a liter in a volumetric flask. A drop of toluene is added as a preservative. Exactly 5, 10, and 15 c.c. of the stock solution of urea are diluted to 50 c.c. in volumetric flasks, making solutions containing 8, 16, and 24 mg. urea nitrogen per 100 c.c., respectively. These and water alone are analyzed exactly as in the procedure for blood, using 0.2 c.c. of the

\*The Cenco-Sheard-Sanford photometer, manufactured by the Central Scientific Co., was used in all these experiments.

standard solution and 0.2 c.c. of urease solution, and carrying out the digestion, deproteinization, heating, and filtering as previously. Two cubic centimeters of the final filtrate are made up with water and 2 c.c. of Nessler's solution to 20 c.c. and read in the photoelectric colorimeter within two minutes. The curve obtained when plotted on semilogarithmic paper is a straight line (Fig. 1). In the determination of blood of high urea concentration, it is more accurate to analyze the diluted blood than to use less of the final filtrate for nesslerization, since, in the latter case, there will be less blank.

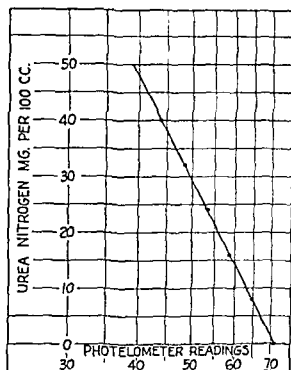


Fig. 1.—The relationship between photometer readings and urea nitrogen concentration.

The standard curve should be repeated with several urease solutions to check on the uniformity of the tablets, for a good portion of the blank comes from the urease. If the tablets are not uniform, it is suggested that a glycerol solution of urease, as recommended by Wren,<sup>10</sup> but containing no acetone, be used. Under any circumstances, it must be recognized that unless the blank of the urease solution is duplicable, photoelectric microanalysis of urea by direct nesslerization is not reliable.

Table I compares the analysis of urea by the proposed method with that by the aeration method of Van Slyke and Cullen.<sup>2</sup> There is close agreement.

TABLE I  
ACCURACY OF DETERMINATION OF UREA  
The results are expressed in mg. per 100 c.c.

SAMPLE	METHOD		ADDED UREA NITROGEN	TOTAL UREA NITROGEN	
	VAN SLYKE AND CULLEN	AUTHORS'		CALCULATED	FOUND
Blood	11.0	11.2	8.0	19.2	19.5
Blood	15.7	15.0	16.0	31.0	31.7
Blood	8.5	8.9	32.0	40.9	40.0
Urine	788	770	400	1,170	1,100
Urine	1,160	1,206	800	2,006	2,040

## SUMMARY

A photoelectric method for the microdetermination of urea in blood and urine by direct nesslerization is offered. Duplicability of the values obtained is brought about by a deproteinization technique which gives clear solutions on the addition of Nessler's reagent, with a minimal and constant blank.

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## PHOTOMETRIC BLOOD SUGAR DETERMINATION BY THE FOLIN-WU METHOD\*

### A NEW SOURCE OF ERROR

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SINCE the application of photometry (optical or electrical) has become so wide as to gradually replace clinical colorimetry, many authors have attempted to adapt previous methods to the new instruments.

One of the most universally accepted methods for blood sugar determination, that of Folin and Wu, has been repeatedly recommended for use with various types of photometers.<sup>1</sup>

Unfortunately, a very important source of error has passed unnoticed: the very marked fading of the color, which takes place immediately after the mixing of the reagents and gradually increases during many hours (Chart 1). It is obvious that, on account of this rapid fading, which seems to have escaped the attention of previous workers,<sup>2</sup> it is absolutely impossible to give any consistent figures for the optical density of the glucose solution (unless all time factors are very rigidly fixed). We have tried to overcome this point by shortening the time necessary to arrive at a final equilibrium, when the fading of the color has practically ceased. We have found that this

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may be obtained by immersing the tubes in a boiling water bath for five minutes after adding the phosphomolybdic reagent (Chart 2). In this way, also, bubbling (which is so troublesome in photometric readings) has been entirely eliminated.

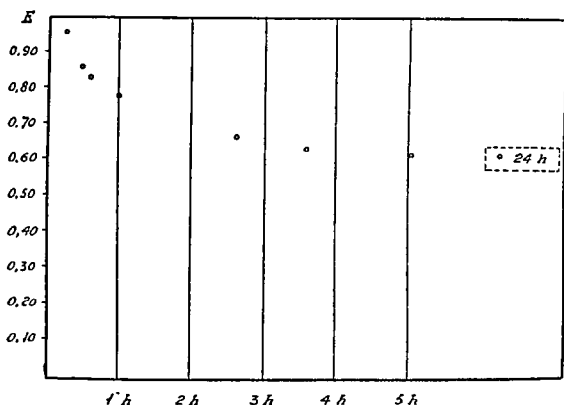


Chart 1.—Fading of color in the course of time, observed in a 0.2 per cent solution of glucose at a depth of 0.5 cm., using a red screen (about  $690 \mu\mu$ ). It is to be noticed that only after twenty-four hours does the phenomenon become insignificant at room temperature.  $E$  = light extinction.

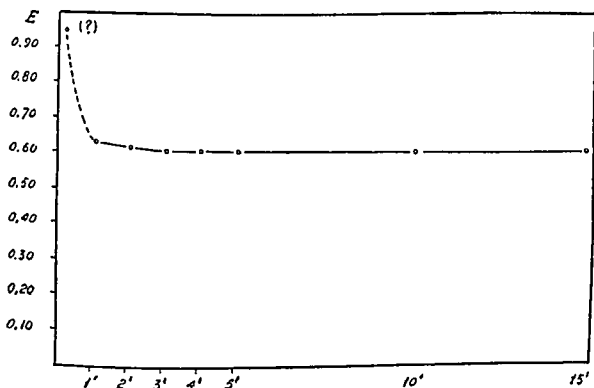


Chart 2.—Loss of color after heating of various duration in a water bath.

Chart 2 shows that after three minutes' heating in a water bath, the color fading has practically reached its final point, so that readings may be made which are easily comparable.

Chart 3 shows the light absorption curve (calculated as extinction) which we have obtained with pure glucose solutions. We obtained a straight line,

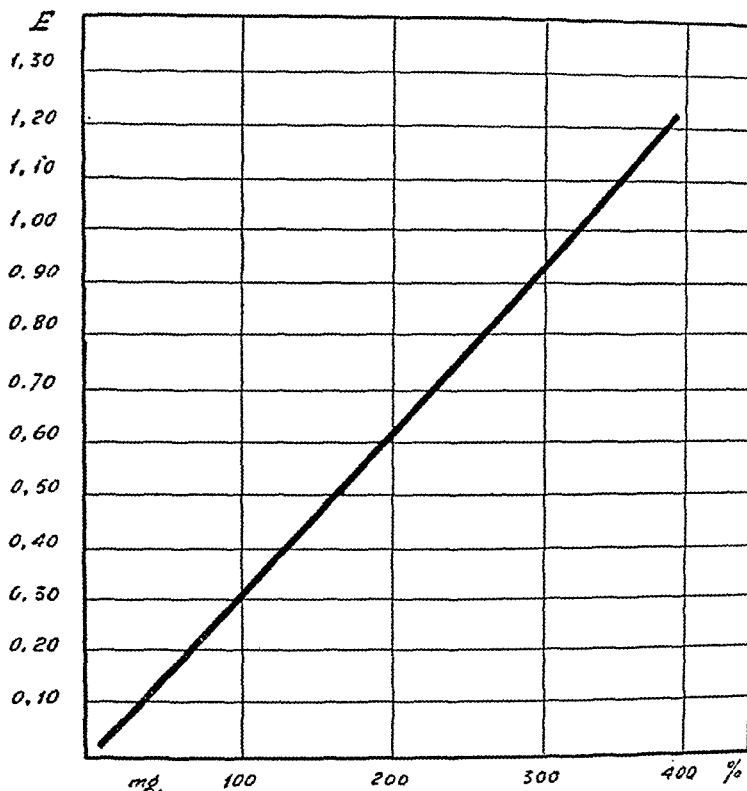


Chart 3.—Color of glucose solutions at various concentrations.

which goes exactly to the zero point. That is to say, there is perfect proportionality between glucose concentration and color production.

Our results are not in accordance with those found in the literature, referring to the alleged nonproportionality of color developed in the Folin-Wu method,<sup>3</sup> but we believe that previous observations are now to be considered as not entirely reliable.

It is obvious that a different chart must be constructed for the use with every optical device in practice, but this is a matter of no real difficulty for any laboratory worker.

#### SUMMARY

It is demonstrated that the color produced in the Folin-Wu glucose determination fades rapidly at room temperature, thus affecting every photometric reading.

It is suggested to stabilize the color by five minutes' heating in a water bath before reading.

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## FURTHER OBSERVATIONS ON THE HEMOLYTIC EFFECTS OF ETHYL AND CAPRYLIC ALCOHOL\*

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THE well-known influence of experimental conditions upon the results of hemolysis studies<sup>1</sup> suggested repetition, with slightly modified technique, of some previously reported observations on the hemolytic effects of ethyl and caprylic alcohol.<sup>2</sup> During these studies, it was noted that caprylic alcohol, despite its marked hemolytic properties, was capable under certain conditions of protecting red blood cells against hypotonic laking. Similar results for an extensive series of aliphatic narcotics have been reported by Yoshitomi,<sup>3</sup> and protection against hypotonic hemolysis has been employed by Sollmann<sup>4</sup> as a criterion of astringent action. The present study includes (a) additional observations on the hemolytic properties of ethyl and caprylic alcohol, and (b) observations on the osmotic resistance of red blood cells in the presence of low concentrations of these reagents.

### (a) Hemolytic Effects of Water and Alcohols

Sixteen samples of fresh beef blood and 5 of hog blood were used in comparing the hemolytic powers of water, ethyl alcohol, and caprylic alcohol. The reagents were added, not directly to blood as in the previous study,<sup>2</sup> but to 0.9 per cent sodium chloride solution in proportions identical with those previously used. Small amounts of blood were transferred to the resulting test mixtures by means of a stirring rod. The suspensions were thoroughly mixed at intervals of five, fifteen, thirty, forty-five, and sixty minutes, and observations for hemolysis were made at five and at sixty minutes. The criterion of hemolysis was transparency of the mixture. All observations were at room temperature (average 25.3° C.; standard deviation 2.4); comparisons were made at equal temperatures.

The results are summarized in Table I, which shows the dilution of hemolytic agent in salt solution at which hemolysis occurred. Included in the table for comparison are the results of adding reagents directly to blood.<sup>2</sup> Data for beef and hog blood were considered together as previous results failed to reveal a significant difference.

Although the results suggest that, in the cases of water and caprylic alcohol, hemolysis was more readily accomplished by adding the reagents directly to blood, this discrepancy may be due to the fact that transparency was of necessity judged in a slightly different manner for the two series of experiments. The difference in medians, where noted, was only one step in the series of dilutions used. The relative hemolytic powers of water, ethyl alcohol, and caprylic alcohol,

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as previously reported and as indicated by the blood series in Table I were approximately 1, 4, and 200, respectively. In the 0.9 per cent NaCl series, the corresponding values were 1, 8, and 200. It is, therefore, doubtful whether the results express a real difference between the two sets of observations. The previously reported slowness of development of full hemolytic capacity by caprylic alcohol was confirmed as noted in Table I.

TABLE I

HEMOLYSIS BY REAGENT DILUTIONS IN BLOOD AND IN 0.9 PER CENT SODIUM CHLORIDE SOLUTION

	TIME	WATER			ETHYL ALCOHOL			CAPRYLIC ALCOHOL		
		MEDIAN		EX- TREMES	MEDIAN		EX- TREMES	MEDIAN		EX- TREMES
	Min.	Dil.	Per cent	Dil.	Dil.	Per cent	Dil.	Dil.	Per cent	Dil.
Blood	5	1	86.7	1,2	3	86.7	3,4	<10	100.0	
	60	1	86.7	1,2	4	86.7	4,5	200	66.7	100,200
0.9% NaCl	5	0.5	71.5	0.5,1	3	100.0		<10	100.0	
	60	0.5	66.7	0.5,1	4	76.2	3,4	100	54.5	100,400

Notes.—Figures in "dilution" columns represent parts of blood or 0.9 per cent NaCl solution per one part of reagent in the test mixture.

Figures in "per cent" columns represent the percentage of samples having the median value.

#### (b) Osmotic Resistance in the Presence of Ethyl and Caprylic Alcohol

Resistance to hypotonic laking of red blood cells from 13 samples of beef blood and 5 samples of hog blood was tested by transferring small amounts of blood, as noted above, to sodium chloride solutions having concentrations of 0.25 to 0.60 per cent. In addition to an untreated control series, four series each of test mixtures containing ethyl and caprylic alcohol were prepared for a given sample of blood. The proportions of alcohols used were (reagent: salt solution): 1:1,000, 1:500, 1:250, and 1:125. Readings were made fifteen minutes after the addition of blood to the test mixtures. Osmotic resistance was measured as the midpoint of the zone between complete hemolysis and imperceptible hemolysis. As only large differences were of interest, no attempt was made to measure red blood cell fragility with great accuracy. All observations were made at room temperature (average 26.0° C.; standard deviation 1.8).

Table II is a summary of the results obtained, including the direction of change in osmotic resistance for individual samples. In the ethyl alcohol series, all dilutions showed identical averages and nearly identical distributions. Moreover, the results were so nearly like the control values that no measurable difference could be postulated on average results. Individual samples showed a definite increase of osmotic resistance in 17.6 per cent of the total possible opportunities, the great majority of samples being unchanged regardless of the dilution of ethyl alcohol used. Of the 9 definite changes recorded, 8 were distributed among the 1:500, 1:250, and 1:125 mixtures, accounting for 13.3 to 23.1 per cent of the samples tested.

In the caprylic alcohol series, averages indicate that the resistance to hemolysis was increased. All the caprylic alcohol averages were significantly<sup>2</sup> lower than the control average, although this was true of the distribution of individual

values only for dilutions lower than 1:500.<sup>6</sup> There was also a statistically significant difference between the average osmotic resistance for the 1:1,000 group and that for the 1:250, indicating a progressive increase in resistance to hemolysis between these two classes. The frequency of definite increases in osmotic resistance among individual samples was most impressive at 1:500 and smaller dilutions, where the number of samples showing change was 87.5 to 93.3 per cent of the samples tested. These frequencies were much greater than the corresponding values for the ethyl alcohol series.

TABLE II

OSMOTIC RESISTANCE OF RED BLOOD CELLS IN THE PRESENCE OF ETHYL ALCOHOL AND CAPRYLIC ALCOHOL

(Readings at 15 minutes)

REAGENT	DILUTION	OSMOTIC RESISTANCE				CHANGE IN OSMOTIC RESISTANCE					
		EXTREMES	AVG.	MED.	S.D.	INCREASE		DECREASE		NO CHANGE	
		<i>Per cent NaCl</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Number</i>	<i>Per cent</i>	<i>Number</i>	<i>Per cent</i>	<i>Number</i>	<i>Per cent</i>
Control		0.42-0.52	0.47	0.47	0.027						
Ethyl alcohol	1,000	0.40-0.53	0.46	0.46	0.055	1	6.3	0	0.0	15	93.7
	500	0.41-0.52	0.46	0.45	0.034	3	18.8	0	0.0	13	81.2
	250	0.40-0.50	0.46	0.46	0.032	2	13.3	0	0.0	13	86.7
	125	0.40-0.52	0.46	0.46	0.038	3	23.1	0	0.0	10	76.9
Caprylic alcohol	1,000	0.40-0.49	0.44	0.44	0.028	5	29.4	0	0.0	12	70.6
	500	0.38-0.46	0.42	0.41	0.024	14	87.5	0	0.0	2	12.5
	250	0.38-0.45	0.41	0.41	0.019	14	93.3	0	0.0	1	6.7
	125	0.38-0.45	0.40	0.40	0.022	10	90.9	0	0.0	1	9.1

Notes.—Figures in "dilution" column represent parts of sodium chloride solution per one part of reagent in test mixture.

S. D. represents standard deviation from the average

Only alterations of osmotic resistance in excess of 0.02 per cent NaCl are recorded as changed in this table.

To ascertain the permanency of the changes just outlined, osmotic resistance was measured for 5 samples of blood at hourly intervals over a period of five hours. Alcohol concentrations were the same as those previously mentioned, with omission of the 1:125 dilution. A smaller number of sodium chloride concentrations was employed. No changes in resistance to hypotonic laking were noted in the ethyl alcohol group during the period of observation. Increase in osmotic resistance occurred in a majority of the caprylic alcohol tests up to the end of the second hour. Beyond this point, 1:500 and 1:250 dilutions began to show definite decreases in osmotic resistance which, at the end of the fourth hour, included all samples. In five hours, two out of five 1:1,000 dilutions still showed increased resistance, while the first decrease for this dilution had just appeared. It is apparent, therefore, that the increased resistance to hemolysis caused by the concentrations of caprylic alcohol here employed was limited in duration, the hemolytic powers increasing in prominence with prolonged contact.

#### SUMMARY

1. The relative hemolytic powers of water, ethyl alcohol, and caprylic alcohol were tested at room temperature over a period of one hour by adding small amounts of blood to various dilutions of hemolytic agent in 0.9 per cent sodium chloride solution. In two-thirds of the samples, water caused hemolysis in dilutions of 2:1. Ethyl alcohol had approximately eight times and caprylic



alcohol about two hundred times the laking power of water. These results were compared with analogous findings for direct additions of hemolytic agents to defibrinated blood. The differences noted were of doubtful significance.

2. Resistance of red blood cells to hypotonic laking was tested at room temperature in the presence of various concentrations of ethyl and caprylic alcohol. Ethyl alcohol caused no changes in five hours at the dilutions employed. Caprylic alcohol in dilutions of 1:500, 1:250, and 1:125 increased the osmotic resistance for at least fifteen minutes in approximately 90 per cent of the samples. Such protective action was diminished in three hours, and only decrease in osmotic resistance occurred after four hours.

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### A NUMERICAL SYSTEM USING COLORS FOR MARKING ALBINO RATS AND MICE\*

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NUMERICAL systems for designating laboratory animals usually involve tattooing, clipping of ears or toes or both, or leg bands, but white rats and mice are so easily marked by putting spots of color on their coats that this is a very popular method. It is especially so if there are objections to maiming the animals by clipping. Most of the laboratories which employ the coloring method are contented with the use of one or two colors and a standard rotation of marked sites, further differentiation being effected by placing the animals in two or more cages in each of which no two animals bear the same mark or combination of marks. This procedure often is not entirely satisfactory. Sometimes it becomes expedient to place a marked animal in a cage already containing an animal with the same mark, thus necessitating additional marking and a change in the records. Moreover, animals which have escaped from their cages are not easily identified. Finally, these markings require cumbersome entries in the records unless numbers are arbitrarily assigned to represent them.

A system of color marking employed recently in our animal room has proved to combine all of the advantages of a numerical system with those of

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the color technique. The improvement in housing, in record-keeping, and in references to experimental animals used in the past, is obvious. Combinations of colors and sites are used and are arranged in an orderly way so that the number which any combination represents may be recognized immediately without reference to a code. Familiarity with the system is acquired usually before 50 animals have been marked.

#### METHOD

The nine different locations on the rat stand for the digits from 1 to 9, and the four colors stand for the column in which these digits are to be found. The nine digits are represented conveniently by the following nine sites:

- |                   |                   |
|-------------------|-------------------|
| 1. Left ear       | 6. Middle of back |
| 2. Right ear      | 7. Left hip       |
| 3. Left shoulder  | 8. Lower back     |
| 4. Back of neck   | 9. Right hip      |
| 5. Right shoulder | 0. No mark at all |

Although the snout is a desirable location on account of its visibility, it is not given a number because the marks placed there vanish too quickly. On the sites listed the colors last for about three weeks and are renewed, as any dye markings must be, when they are about to become indistinct. The four colors, yellow, red, green, and purple, show whether the digits belong in the ones, tens, hundreds, or thousands column, respectively. This order is chosen according to the permanence of the stains on the coats of the animals; the numbers in the ones and tens column are usually the most significant and are, therefore, assigned the most lasting colors. The practical application of the system is shown best by the examples in Table I. Obviously, a number in which the same digit occurs more than once (55, 1232, 6686, etc.) cannot be represented, for that would entail the application of two colors to the same site. It is for this reason that not all of the 9999 numbers are available. The same digit appears two or more times in 4491 numbers, thus leaving 5508 which are suitable for use.

TABLE I

MARK	PURPLE (n---)	GREEN (-n--)	RED (--n-)	YELLOW (---n)
3	Left shoulder	Left shoulder	Left shoulder	Left shoulder
30				
300				
3000				
34				Back of neck
43	Back of neck	Back of neck	Left shoulder	Left shoulder
403				Left shoulder
430				Right ear
432				Left ear
4321				Left hip
8967	Lower back	Right hip	Middle of back	

The colored marks on the animals can be produced by painting on the following solutions: (1) yellow, by a saturated aqueous solution of picric acid; (2) red, by Ziehl's carbol-fuchsin solution; (3) green, by a 2 per cent solution

of malachite green in 50 per cent alcohol; and (4) purple, by a saturated alcoholic solution of gentian violet diluted with thirty volumes of 50 per cent alcohol. The purple and green solutions probably may be diluted considerably before they fail to give satisfactory results. One ounce widemouthed bottles stoppered with corks in which camel's-hair brushes have been inserted make convenient containers for these solutions. The bottles are mounted in a row in a wooden block. None of these dyes has been found to be poisonous to the animals.

#### SUMMARY

A numerical color system for marking white rats and mice is described. With the application of one to four colors on one to four of nine locations on the animal there become available about fifty-five hundred different combinations which may be designated numerically.

### METHOD OF SIMULTANEOUS FIXATION AND DECALCIFICATION OF BONE\*

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THE method effecting simultaneous fixation and decalcification of bone has been successfully employed in our laboratory for a period of five years. Because of the corrosive sublimate fixation, the resulting preparations are suitable for the application of the majority of the special staining procedures. With this method of decalcification, tissue distortion is minimal, and the staining capacity of the nuclei is not diminished.

#### *Decalcifying-Fixing Fluid:*

Alcohol, 95 per cent	50 c.c.
Formaldehyde	40 c.c.
Nitric acid	5 c.c.
Corrosive sublimate	10 Gm.
Trichloroacetic acid	30 Gm.
Distilled water	400 c.c.

1. Dissolve corrosive sublimate in 300 c.c. of water *with the aid of heat*. Cool. This is solution No. 1.

2. Dissolve trichloroacetic acid in 100 c.c. of water, add nitric acid, alcohol, and formaldehyde. This is solution No. 2.

3. When solution No. 1 is cool, add solution No. 2.

4. The finished stock solution will keep indefinitely in the icebox. At room temperature the mercury tends to precipitate after three or four weeks.

\*From the Pathological Laboratory, Veterans Administration Hospital, Published under R. & P. 6727, U. S. Veterans Administration.  
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## METHOD

1. Cut sections of fresh bone, not more than 8 to 10 mm. in thickness with a scroll saw.

2. Place sections in decalcifying-fixing solution (at least 100 times as much fluid as tissue) and place in an incubator at 37° C. The entire process of decalcification is carried on at this temperature.

3. Replace the decalcifying-fixing fluid daily until tissue is soft. The degree of decalcification may be tested by palpation or by cutting an extremely thin slice with a sharp knife. The common practice of piercing the tissue with a needle frequently breaks the architecture in the most interesting portion of the section.

4. When the tissue is soft, place in running water for twenty-four hours. (We have found this sufficient; however, 1.5 to 2 per cent ammonia water may be used for twelve hours, followed by running water for twenty-four hours.)

Place tissue in:

80 per cent alcohol for two hours

95 per cent alcohol for six hours

Absolute alcohol for from sixteen to twenty-four hours

Xylol for from five to ten minutes

Oil of cedar (clearing) for twenty-four hours

Xylol (two changes) for thirty minutes

Paraffin 56° to 58° (3 changes) for from four to six hours in  
all.

Embed.

If the following procedure is employed before staining the intensity of the nuclear stain is increased: (1) Immerse mounted sections (after removal of paraffin) overnight in Bouin's fluid. (2) Wash in running water for from fifteen to thirty minutes.

With certain sections of bone altered by pathologic changes, such as tumor infiltrations, there is danger of maceration when an attempt is made to saw them into small pieces for decalcification. Such tissue may be placed in the decalcifying-fixing fluid for two days or more for partial decalcification. They may then be cut with a sharp knife into sections of suitable size. Decalcification is then continued.

The average piece of bone decalcifies in from three to five days; if it requires longer than seven days, nuclear staining is impaired.

# CULTURAL DIAGNOSIS OF TUBERCULOSIS USING BORDET-GENGOU AND LÖWENSTEIN MEDIA\*

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CULTURAL diagnosis of tuberculosis has been studied by many investigators with a view of substituting culture for guinea pig inoculation. Although a great deal of work has been done and many excellent media have been devised for this purpose, there is still considerable difference of opinion with regard to the advisability of relying on culture alone.

In a recent review of the literature, Murphy and Duerschner,<sup>1</sup> and Guggenheim and Finkelstein<sup>2</sup> summarized the results obtained by various investigators in comparative studies of culture and guinea pig inoculation. These may be divided into three groups: (1) Equally good results were obtained on culture and guinea pig inoculation. (2) Better results were obtained on culture than on guinea pig inoculation. (3) Better results were obtained on guinea pig inoculation than on culture.

In considering the value of any diagnostic procedure, one must take into consideration not only the accuracy, but also the practicability of the method. Although guinea pig inoculation is undoubtedly an excellent method of detecting small numbers of tubercle bacilli, this method is limited in its application because of the expense involved and also because comparatively few of the small laboratories are equipped to carry animals. Culturing on a suitable medium is relatively inexpensive and can be carried out in any laboratory that has a trained technician. The results obtained on culture by a large number of investigators have been so satisfactory that the adoption of culture as a diagnostic procedure seems to be justified.

The question of an optimum medium for cultural diagnosis is still a matter of opinion. A number of media are available, and good results have been reported with a large number of them.<sup>1, 2</sup> The choice of any particular medium would depend largely on the preference of the individual worker.

One of us (L. M.) reported in 1932<sup>3</sup> the results of a study of the growth of the human and bovine types of tubercle bacilli on various media. We found at that time that of the media tested, the Bordet-Gengou<sup>4</sup> and the Löwenstein<sup>5</sup> egg medium gave the best results. In a second publication<sup>6</sup> in 1934 we reported the results obtained in a comparative study of culturing sputa on the above media and simultaneous inoculation of guinea pigs. Of 233 specimens tested at that time, 18 were positive on the Bordet-Gengou medium, 13 on the

\*From the Bureau of Laboratories, New York City Department of Health, Dr. R. S. Muckenfuss, Director.

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Löwenstein medium, and 15 on guinea pig inoculation. Based on these findings, we have since then substituted culture on the Bordet-Gengou and Löwenstein media for guinea pig inoculation.

We have cultured since our last publication a variety of specimens which included sputa, tissues, pus, pleural fluids, spinal fluids, urines, and other pathologic materials. Most of the specimens came from patients who had been repeatedly negative on routine smear examinations. Each specimen was examined by smear and only those were cultured that were negative on careful smear examination.

#### METHOD OF CULTURE

*Sputum.*—The sputum was poured into a Petri plate and examined for cheesy particles which, if present, were collected by means of a broken-tip pipette or a heavy wire loop, and two thick smears were made. The smears were stained by the Ziehl-Neelsen method, using brilliant green 1:1,000 dilution as a counterstain, and were examined thoroughly for acid-fast bacilli. We found in a previous study<sup>7</sup> that by selecting the material in the manner described the result was the same as concentrating the sputum. If the specimen was negative on smear examination, it was cultured as follows.

The specimen was placed in a sterile 40 c.c. centrifuge tube containing glass beads, and an equal amount of 5 per cent oxalic acid was added (Corper and Uyei method<sup>8</sup>). The tube was stoppered with a sterile solid rubber stopper, and the mixture was incubated at 37° C. for forty-five minutes. During the incubation the mixture was shaken vigorously at frequent intervals. A few drops of bromthymol blue were added after incubation, and the reaction was adjusted to pH 6.8-7.0 with 10 per cent sodium hydroxide. The specimen was then centrifuged at high speed for one hour. The supernatant fluid was decanted, and the sediment was suspended in about 5 c.c. of sterile distilled water, filtered through a thin layer of sterile cotton and inoculated on several plates of the Bordet-Gengou and Löwenstein media. The plates were incubated at 37° C. for two to three days without sealing them to allow the excess moisture to evaporate. They were then sealed with Scotch photographic tape and re-incubated for five to six weeks. The cultures were examined microscopically once a week, using a low power microscope.

*Pus.*—If the pus was very thick, a suspension was made in sterile distilled water; if not, it was used undiluted. A portion of the specimen was inoculated on one plate each of the Bordet-Gengou and Löwenstein media, also on a blood plate and in blood broth in order to detect other organisms. If there was no growth after forty-eight hours' incubation, several more plates of the Bordet-Gengou and Löwenstein media were inoculated with the remainder of the specimen which was kept refrigerated. If the specimen showed contamination on the preliminary cultures, it was treated with oxalic acid in the manner described for sputa.

*Pleural Fluids, Spinal Fluids, and Other Internal Effusions.*—These were usually condensed by centrifugation for one hour at high speed. The sediment was examined by smear, and, if the result was negative, they were cultured as described under "Pus."

*Tissues.*—The surface of the tissue was seared and the infected areas were removed and macerated with sterile scissors. A heavy suspension of the tissue was made in sterile distilled water by grinding it in a sterile mortar and gradually adding the water. The suspension was filtered through sterile cotton to remove large particles of tissue and was tested for contaminants as described under "Pus." If contaminants were present, the remainder of the suspension was treated with an equal amount of 4 per cent sodium hydroxide\* and was incubated at 37° C. for forty-five minutes. The reaction was then adjusted to pH 6.8-7.0 with 10 per cent hydrochloric acid and cultured on several plates of Bordet-Gengou and Löwenstein media.

*Stomach Contents and Urine.*—The specimens were centrifuged at high speed for one hour. The sediment was suspended in sterile distilled water and treated the same as sputum.

*Throat Swabs.*—The swab was placed in a tube containing a small amount of sterile distilled water, and an equal amount of oxalic acid was added. The tube was shaken vigorously and incubated at 37° C. for forty-five minutes. The liquid was filtered through sterile cotton and cultured.

We have cultured in this manner 1,108 specimens and the results are summarized in Tables I and II.

TABLE I  
SPECIMENS NEGATIVE ON SMEAR EXAMINATION

SPECIMEN	TOTAL CUL- TURED	BORDET-GENGOU MEDIUM				LÖWENSTEIN MEDIUM				TOTAL POSITIVE ON BOTH MEDIA	
		POS.	NEG.	CON- TAM.	% POS.	POS.	NEG.	CON- TAM.	% POS.	NO.	%
Sputum	716	82	590	44	11.4	73	617	26	10.1	96*	13.4
Tissues	109	26	78	5	23.8	21	84	4	19.2	27	24.7
Pus	39	19	19	1	48.7	21	18	0	53.8	23	58.9
Pleural fluid	149	61	85	3	40.9	61	88	0	40.9	72	48.3
Spinal fluid	6	3	2	1	50.0	4	2	0	66.6	4	66.6
Miscellaneous fluids†	8	0	7	1	0	0	8	0	0	0	0
Stomach contents	14	0	11	3	0	2	10	2	14.2	2	14.2
Throat swabs	8	0	8	0	0	0	8	0	0	0	0
Urine	59	8	45	6	13.5	6	49	4	10.1	10	16.9
Total examined	1,108	199	845	64	17.9	188	884	36	16.9	234	21.7

\*There were some positive on one medium and negative on the other. Therefore, the total number of positive is greater than on either one of the media.

†Abdominal fluid, knee fluid, bursitic fluid, scrotal fluid, hydrocele fluid.

The largest percentage of positive results was obtained on culturing pus, pleural fluids, and spinal fluids, followed, in the order given, by tissues, urines, and sputa (see Table I). If one takes into consideration that all of these specimens were negative on smear examination, the results seem highly satisfactory.

Growth of tubercle bacilli appeared on both media within two to three weeks; the average period of incubation was seventeen to eighteen days. In a few instances, growth appeared in nine to ten days. Occasionally when the growth was very scant—one to three colonies on all the plates inoculated—they

\*We have changed recently to 5 per cent oxalic acid with equally good results.

were not detected until four or five weeks of incubation. For this reason, no specimen was reported negative until the culture was incubated five to six weeks. A positive result was reported to the physician as soon as typical growth appeared, which was confirmed by smear examination. When the growth was atypical but the smear of the colonies showed acid-fast bacilli, a preliminary report was sent to the physician and a final report was made after the culture was tested for virulence. All cultures of acid-fast bacilli that were obtained from urine were tested for virulence, although most of them were typical on colony morphology.

The growth of tubercle bacilli on the Bordet-Gengou medium was, in many instances, detected earlier than on the Löwenstein medium. The young colonies on the Bordet-Gengou medium are pearly in appearance and stand out more clearly against the dark background of the medium.

TABLE II

COMPARISON OF THE GROWTH ON THE BORDET-GENGOU AND THE LOWENSTEIN MEDIA OF 234 POSITIVE CULTURES

SPECIMENS	BORDET-GENGOU MEDIUM	LOWENSTEIN MEDIUM
153	Positive	Positive
36	1 to 10 colonies	Negative
10	Fairly good growth	Negative
18	Negative	1 to 3 colonies
1	Negative	Fairly good growth
16	Contaminated	Positive*
Positive on the Bordet-Gengou medium		199
Positive on the Löwenstein medium		188

\*Six of these specimens had 1 to 4 colonies, 1 had scant growth, and 9 had good growth.

Comparing the results obtained with the two media, we find that more specimens were lost through contaminations on the Bordet-Gengou than on the Löwenstein medium (Table I). This is not surprising since the Bordet-Gengou medium has no inhibitory substances, whereas the Löwenstein medium contains a dye which restricts the growth of some of the contaminants. However, in spite of the greater loss of specimens through contaminations, the total number of positives on the Bordet-Gengou medium was greater than on the Löwenstein medium (see Tables I and II). If the pathologic material for culture could be obtained without undue contamination, or if the specimens could be cultured soon after they are obtained, the Bordet-Gengou medium could be used alone with excellent results.\* We cultured 279 miscellaneous specimens from October, 1937, through December, 1938, and of this number we lost only 6 specimens through contaminations.

The use of both media had the advantage that in some instances growth was obtained on the Löwenstein medium when the Bordet-Gengou medium was negative or contaminated (Table II). Of 81 specimens that were positive on only one of the two media, 46 were positive on the Bordet-Gengou and negative on the Löwenstein medium, 19 were positive on the Löwenstein medium and negative on the Bordet-Gengou medium, and an additional 16 specimens were positive on the Löwenstein medium when the Bordet-Gengou medium was over-

\*Gernez, Crampon, and Graux<sup>9</sup> confirmed our findings with regard to the value of the Bordet-Gengou medium in the isolation of human and bovine tubercle bacilli.



grown by contaminants. Thus an additional 35 specimens were found positive by using the Löwenstein medium in addition to the Bordet-Gengou medium. It would, therefore, be advisable to use both media if they are available. The use of more than one medium has been recommended by Whitehead<sup>10</sup> recently when he tested several different media. He found that Petraghani's medium gave much better results than the Löwenstein medium. In our hands, the results on both media were approximately the same.

We found the Bordet-Gengou medium especially advantageous because it is an inexpensive medium and both the human and bovine types of tubercle bacilli grow well on it. It is easily prepared and can be kept in the icebox almost indefinitely if it is stored without adding the blood. It may be stored for several weeks when the blood is added, but after that it tends to become dry and is less favorable for the growth of tubercle bacilli. This medium was prepared according to the original formula<sup>4</sup> until about one year ago, when we began using the modification of the Bordet-Gengou medium that was recommended by the Committee of Standard Methods of the American Public Health Association.<sup>11</sup> We found that the growth of tubercle bacilli on the modified Bordet-Gengou medium was more luxuriant than on the original medium provided approximately 30 per cent of normal blood was added to the medium. We use normal horse blood because it is readily available in our laboratories.

The Löwenstein medium was prepared according to the original formula.<sup>5</sup> The only change made was that we use 1.5 per cent malachite green instead of 2 per cent Congo red.

#### SUMMARY

Cultural diagnosis of tuberculosis instead of guinea pig inoculation has been employed in our laboratories for the past five and one-half years. Only those specimens were cultured that would ordinarily have to be injected into guinea pigs, that is, those that could not be diagnosed on smear examinations.

A total of 1,108 specimens of sputa, tissues, pleural fluids, pus, urines, and other pathologic materials were cultured. Each specimen was examined by smear, and those that were negative were cultured on the Bordet-Gengou and Löwenstein media.

The largest percentage of positive results was obtained on culturing pleural fluids, spinal fluids, and pus—48.3 to 66.6 per cent. Tissues were positive in 24.7 per cent, sputa in 13.4 per cent, and urines in 16.9 per cent.

Positive cultures were obtained within two to three weeks, with an average of seventeen to eighteen days. Occasionally a positive culture was obtained in nine to ten days, and in a few instances, where the growth was very scant, it was not detected until the culture was incubated for four to five weeks. If the cultures were negative after five to six weeks' incubation, no growth was obtained on longer incubation. We, therefore, made the period of incubation five to six weeks.

More positive cultures were obtained on the Bordet-Gengou than on the Löwenstein medium, but occasionally growth of tubercle bacilli was obtained on the Löwenstein medium when the Bordet-Gengou medium was negative or overgrown by contaminants.

## CONCLUSIONS

1. Cultural diagnosis of tuberculosis on the Bordet-Gengou and Löwenstein media has been found practicable, and the results obtained compare favorably with the results that we obtained formerly on guinea pig inoculation.

2. The Bordet-Gengou medium has the special advantage that it is inexpensive. It can be kept for a long time, and tubercle bacilli of both the human and the bovine type grow well and characteristically on it. Its main disadvantage is that it has no inhibitory action on contaminants that resist acid or alkali treatment. It is, therefore, advisable to use another medium that has inhibiting substances in conjunction with the Bordet-Gengou medium. The Löwenstein's medium has been used with good results. If the pathologic material could be obtained without undue contamination, the Bordet-Gengou medium could be used alone with excellent results.

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## COVER SLIP DISPENSER\*

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THE machine illustrated in Fig. 1 was designed as an aid in handling cleaned cover slips. The cover slips are ejected singly in a position to be easily grasped by the edges without smearing the cleaned surfaces.

In essence the machine is composed of a plunger of correct thickness, running on a horizontal plane through a box containing cover slips, the edges of the box being raised sufficiently in the direction of motion of the plunger to allow only one cover slip to be pushed out of the box by the forward motion of the plunger. From the illustration, the construction can be easily followed.

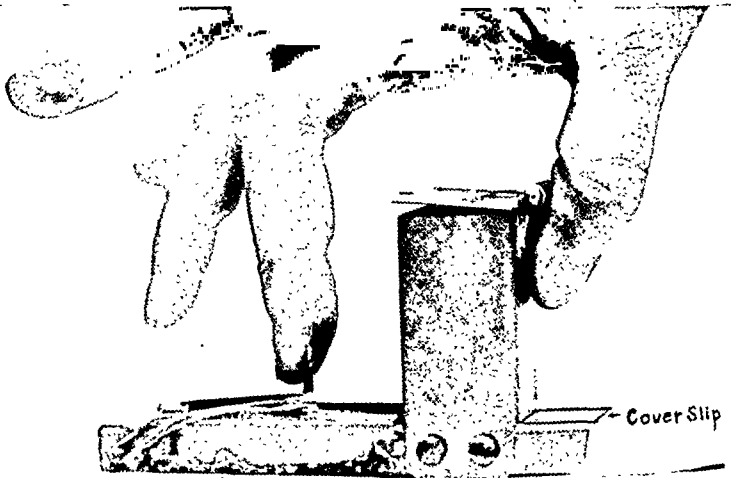


Fig. 1.

The box holding the cover slips can be cut out and bent to shape from scrap pieces of light gauge stainless steel or monel metal from a tinner's shop. The edges under which the plunger and cover slip run must, of course, be parallel with the base. The bolt holes in the box should be elongated in order that the slit for the emerging cover slips can be adjusted to the correct height. The inside dimensions of the box must not be much larger than the cover slip, but the height is a matter of choice. Not visible in the illustration is an angled extension of one side of the box, about two-thirds of the height from the base, to facilitate insertion of the cleaned slips. A hinged cover is attached to the box to protect the slips from dust.

The base must be rigid and conform to the size of the box. It is perhaps best made of a piece of channel bar of the correct size. The top surface of the base must be square and smooth.

\*From the Laboratory of the C. V. Memorial Hospital, Johnstown.  
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The plunger, of which only the knob is visible, is the 0.006 inch blade of a thickness gauge, soldered at the back end to a heavier piece of metal. The front of the blade, the width of the box, must be free to slide through the box. The blade must be sufficiently long and not too pliable. The heavier block lends rigidity to the plunger and is arranged so that its front edge against the box acts as a stop to forward motion. A screw in the proper place in the back of the base is a stop to backward motion. On the heavier block is soldered a knob to facilitate moving the plunger.

The runway is merely two pieces of angle adjusted to fit the heavier block of the plunger and soldered on the base. The solder can doubtless be applied more smoothly than that shown in Fig. 1. There should be comparatively little horizontal or vertical play in the plunger.

A pin is soldered on either side of the back and the base as an anchor for a spring (rubber band) which returns the plunger to position for another stroke.

By adjusting the slit at the base, either No. 1 or No. 2 cover slips can be dispensed.

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## THE IDE TEST FOR SYPHILIS

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**F**OLLOWING the publication of a report on a simple test for syphilis, as devised by Dr. T. Ide and Dr. S. Ide<sup>1</sup> in this JOURNAL August, 1936, an attempt has been made to evaluate this test in general practice.

The Ides state that the test has special advantages in that with only one drop of blood and a simple technique the result can be read clearly by the coloring under the microscope. It can be made in a few minutes' time in the examining room. They state that the accuracy of the test compares favorably with the Wassermann, Meinicke, Sachs-Georgi, Kahn, and Murata. The test can be performed with blood serum, whole blood, spinal fluid, and serous exudate.

The desirability of a simple test for syphilis is apparent. A test that can be done quickly, accurately, and inexpensively would be of great value to the practicing physician without a large laboratory. It would certainly be a boon to the physician practicing in the country, who, though having access to laboratories miles away, would find a test of this type exceedingly helpful. The test described herein approaches in simplicity a routine urinalysis.

A few evaluations of this test have appeared in the literature, but the results as given are somewhat conflicting. Most of the reports seem to have come from laboratories where standard serologic tests are also done. Thus, if this test is of value, its agreement with standard tests should be higher than the

results obtained by one who is untrained in serologic work. The test as outlined, however, is for the benefit of the latter worker as well as for serologic experts.

The *British Medical Journal*,<sup>2</sup> in an editorial, state that the test seems worthy of trial. They raise the warning that it is doubtful whether a serologic test for syphilis is yet sufficiently foolproof to be removed from the hands of the experienced pathologist. They point out that even the simplest tests have pitfalls, not only in the setting up of the reaction, but also in the reading and interpretation of the results.

In 1,262 tests the Ides<sup>1</sup> obtained 1,126 negative Wassermann reactions, and 1,140 negative Ide tests. One hundred nineteen persons gave strongly positive Wassermann reactions, and 116 gave strongly positive Ide reactions. They obtained no false positive Ide tests.

Rein and Hazay<sup>3</sup> have modified the Ide test by inactivating sera at 56° C. for thirty minutes. They have also used slides similar to those used in the Kline test. Of 482 nonsyphilitic persons whose sera gave negative reactions with the Kline diagnostic and exclusion test, 92.11 per cent gave negative reactions with the Ide test. Of 555 syphilitic sera, there were 2.88 per cent disagreements. They conclude that the Ide test has been found to be somewhat less sensitive and less specific than the Kline heated serum tests.

Flood and Moyer<sup>4</sup> found that 51 per cent of the Ide tests agreed with the standard tests performed on 565 specimens of syphilitic sera. Of the total 822 Ide tests they performed, there was agreement with controls in 61 per cent. They are at a loss to explain these results. They conclude that the Ide test is inadequate for the serodiagnosis of syphilis.

Breazeale, Greene, and Harding,<sup>5</sup> in a large series of cases (1,000), state that the Eagle, Ide, Kahn, Kline, and Laughlen tests are equally reliable if the user adheres to the recommended technique.

Demanche and Segol<sup>6</sup> believe that the Ide test is less sensitive than the Wassermann, Kahn, and Meinicke, and is less precise. They believe that the test should be checked by more exact methods. (Series 295 cases.)

Our report is based on 210 tests carried out in a small office, with no special laboratory space. Routine blood counts, urinalyses, and smears are about the only laboratory procedures regularly done in the office. Equipment necessary to conduct the test is inexpensive or already in the physician's possession.

We assembled two 1 c.c. pipettes, graded in hundredths of a cubic centimeter, two hanging-drop slides, cover slips, medicine droppers, the antigen, a small test tube for the diluted antigen, and small quantities of 2.5 per cent and 3.5 per cent saline solution. We used a microscope for reading the test.

The procedure used has been outlined in the original article and has been used on whole blood, serum, and spinal fluid. The majority of the tests have been carried out, using serum which has been separated from the cellular elements either by centrifuging or by standing.

The antigen used was purchased commercially or used from a supply sent by the Ides. The more recent tests have frequently been carried out using both antigens. The antigen has been kept at room temperature. Although

the method for preparing the antigen is described, its preparation seems too involved and time-consuming for the individual who might best use the test.

In brief, the procedure of the test has been to prepare the diluted antigen (0.2 c.c. of stock antigen with 0.6 c.c. of 2.5 per cent saline solution) immediately before it is to be used. When we have had only a very few tests to make at one time, we have used 0.1 c.c. of antigen to 0.3 c.c. saline, with no variation in the results when checked by the full quantity of antigen and saline solution.

A drop of whole blood is placed on the hanging-drop slide and mixed with a drop of 3.5 per cent saline solution, and then spread over the depression. To this is added a drop of the diluted antigen. If blood serum or spinal fluid is used, the dilution with saline is omitted. The slide is then shaken so as to thoroughly mix the antigen and the blood or serum. This should be read at once. We have not used cover slips on the preparations as they seem to be in the way, and the amount of drying and evaporation is slight during the time necessary to mix and read the preparation.

A total of 210 Ide tests have been made and checked against Wassermann, Kahn, and Kline tests. The majority of the Wassermann and Kahn tests have been made in the laboratories of the Knoxville General Hospital or in the laboratories of the Bureau of Health of Knoxville. A few tests have been made by private laboratories. Sera have been obtained from these laboratories following the performance of these standard tests, and the Ide test then made. I have adhered closely to the technique originally outlined as the test could hardly be simplified. The results obtained are shown in Table I

TABLE I

Ide	Negative	Wassermann	Kahn	Kline	Negative
163		86	75	6	
Ide	Positive	Wassermann	Kahn	Kline	Positive
24		17	6	1	
Ide	Negative	Wassermann	Kahn	Kline	Positive
8		5	3		
Ide	Positive	Wassermann	Kahn	Kline	Negative
8		4	4		
Ide	Doubtful	Wassermann	Kahn	Kline	Doubtful
2		1	2		

## SUMMARY

1. The Ide test for syphilis is considered chiefly from the standpoint of the physician without extensive laboratory facilities.

2. A brief review of the literature is given.

3. The results of 210 Ide tests as compared with an equal number of standard serologic tests are given. Total agreement was 92.39 per cent. The number of false positive Ide tests was 4.57 per cent. The number of false negative Ide tests was 25 per cent. Eight negative reactions in a total of 32 positive Wassermann or Kahn reactions are a large margin of error. I do not believe the test sufficiently sensitive and specific to be wholly relied upon for the serodiagnosis of syphilis.

I am indebted to Dr. Thomas B. Drinnen for his assistance in part of this work.

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## THE CONCENTRATION OF TUBERCLE BACILLI FROM SPINAL FLUID BY MEANS OF CHEMICAL FLOCCULATION AND LIPOID SOLVENTS\*

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WE HAVE shown that the collection of tubercle bacilli from aqueous suspensions, sputum,<sup>1</sup> or urine<sup>2</sup> by direct centrifugation is much less efficient than might be expected in view of the relative ease with which other bacteria are sedimented by this method. For example, after centrifugation of slightly positive sputa for one hour, chemical flocculations revealed that many bacilli remained in the supernatant fluids. The incorporation of 0.2 per cent alum in the usual 4 per cent sodium hydroxide digester for sputum, and flocculation of the alum by neutralization prior to centrifugation for five minutes, increased the effectiveness of concentration from sputum.

In view of the importance of concentration methods in the diagnosis and control of tuberculosis, these studies have been extended to an examination of the collection of tubercle bacilli from spinal fluids by means of several of the methods in current use, and especially by means of chemical flocculation and lipid solvents.

### METHODS

Two kinds of material were studied: a series of "synthetic" tuberculous spinal fluids prepared for the study of various technical problems, and a series of spinal fluids submitted for bacteriologic confirmation of the clinical diagnosis from persons suspected of tuberculous meningitis.<sup>†</sup>

The synthetic tuberculous spinal fluids‡ were prepared by addition of known amounts of clump-free, precounted suspensions of human type (H-37)

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†We are indebted to the Clinical Laboratory, Dr. E. Clarence Rice, and the resident staff of the Children's Hospital, for these specimens and for information from the hospital records of each case.

‡The usefulness of such materials in studies of this nature has not been sufficiently appreciated. The bacillary content of the "synthetic" tuberculous spinal fluid is known, and a measure of the actual degree of concentration may be obtained by quantitative counting. The suspension, free of clumps, provides the most rigorous test for the collecting power of any method. At the same time one avoids the errors introduced by the chance distribution and simultaneous testing of any number of methods. By means of this experimental approach it is possible not only to select the better methods but also to indicate the increased possibility of demonstrating the bacilli by a concentration factor.

tubercle bacilli to pooled normal spinal fluids. The method of suspending the bacilli, the separation of the individual bacilli from the clumps, and the preliminary standardization by turbidity, have been given elsewhere.<sup>1</sup> These suspensions were heated at 80° C. for fifteen minutes in the presence of 0.2 per cent formaldehyde. This procedure did not alter the microscopic counts. The heated suspensions were stored in the icebox for several weeks without loss of acid-fastness and with negligible clumping. Nevertheless, each suspension was counted prior to its use in each experiment.

The microscopic method of counting the bacilli consisted of mixing the bacillary suspensions, or the sediments after concentration, with an equal volume of alum milk (Difco dehydrated milk 5 per cent, alum 0.5 per cent, formaldehyde 0.2 per cent) and spreading 0.02 c.c. of this mixture on carefully standardized areas of 2 by 2 cm. on clean glass slides in duplicate. The films were dried, fixed with heat, and stained by immersion in Ziehl-Neelsen carbolfuchsin at 37° C. for thirty minutes. Staining, decolorization, washing, and counter-staining (methylene blue or picric acid) were carried out in jars to avoid the slide losses induced by the action of running water on the films. The stained films were examined by counting the bacilli in ten or more fields selected in orderly fashion along each diagonal of the films, so that 40 or more fields were observed in each determination from the two films. Data on the distribution of bacteria in such films and on the quantitative aspects of microscopic counting will be presented elsewhere.<sup>4</sup>

In the various experiments the original suspensions revealed from two to six bacilli per microscopic field before being added to the pooled spinal fluids in such amounts that the final dilution was 1:100. The number of bacilli which might be anticipated per field in the unconcentrated fluids (0.02 to 0.06 bacilli per field) was regarded as unity. The average number of bacilli counted per field after concentration represented a multiple of the value of unity in each experiment. This multiple, to be designated as a *concentration factor*, indicates the degree of concentration. It affords a simple means of comparing the effectiveness of different methods. All clumps were scored as single bacilli.

For each experiment a sufficient amount of the "synthetic" tuberculous fluid was prepared to provide 2 c.c. portions to be concentrated by each procedure. These tests were carried out in small tubes in order to simulate the conditions of manipulation and centrifugation encountered in small clinical specimens. The methods of concentration are classified and summarized as follows.

#### A. Centrifugation:

1. Cheer's alcohol method<sup>5</sup>—Addition, by drops, of one-third to one-half volume of alcohol and, if needed, a small amount of egg albumen to produce precipitation. Centrifugation as described under Direct centrifugation.
2. Direct centrifugation—Centrifugation for one hour at a relative centrifugal force of 500 times gravity; sediments smeared in approximately equal volume of alum-milk fixative.



These procedures are widely used at the present time and, in this sense, may be regarded as controls.\*

B. *Flocculation Methods*: Centrifugation for five minutes; sediments mixed with an equal volume of alum-milk fixative.

1. Alum—The 2 c.c. specimen was pipetted forcibly into 0.25 or 0.5 c.c. of 1 per cent aqueous solution of potassium alum. Flocculation was prompt but slight.
2. Calcium phosphate—0.1 c.c. M/10 calcium chloride and then 0.1 c.c. M/15 dipotassium phosphate were added to the fluid during constant shaking.
3. Ferric chloride—1 per cent aqueous solution of ferric chloride was added drop by drop (with continuous shaking) until flocculation was observed.

C. *Lipoid Solvents*: Centrifugation for five minutes; the collected materials could not be mixed with milk fixative.

1. Chloroform—0.05 c.c. was added to each 2 c.c. specimen. The tube was shaken violently for ten minutes; the entire sediment beneath the discarded supernatant was used.
2. Xylol—Procedure as above; the supernatant creamy layer was used.

## RESULTS

Inasmuch as the studies in the "synthetic" fluids revealed objectionable features in several of the foregoing methods, these difficulties will be considered first in order to limit the discussion to the more useful procedures.

Cheer's method was more troublesome than direct centrifugation in that several alternate additions of alcohol and egg albumen were required. The results were frequently inferior, perhaps because of the dilution of the sample by the reagents. It has been noted that the collection time for each of the centrifugation methods was one hour.

The calcium phosphate method was disqualified because two separate reagents were required and because the sediments did not smear well when used alone or with the milk fixative. Sudden, voluminous precipitation occurred frequently with ferric chloride. The large sediments interfered with effective concentration. Furthermore, the 1 per cent solution of ferric chloride deteriorated on storage.

The xylol flotation method apparently collected fewer bacilli than the chloroform, possibly because the xylol cream could not be skimmed from the centrifuged sample with ease or completeness. A satisfactory rating could not be assigned. Since the chloroform sediments from a clear solution, such as spinal fluid, were not objectionable because of debris, smeared well, and could be recovered completely from the tubes after removal of the supernatant fluid, this method was preferred to the xylol flotation. In the case of either lipoid solvent, the use of milk fixative was not feasible and, apparently, not

\*Isolated observations on the possibility of demonstrating tubercle bacilli in the "web" have been discouraging.

necessary. A peculiarity of the lipid solvent methods was that the collected materials could not be smeared if collection was made from formalinized spinal fluids in the neutral or alkaline range of pH values. This difficulty was overcome by the addition of two drops of bromeresol green color indicator and sufficient N/1 hydrochloric acid to produce a green or yellow color (pH approximately 4.5 to 4.0).

In Table I are shown the average concentration factors for the two most satisfactory methods (alum and chloroform) and the control method of direct centrifugation. The values are based on four of the experiments in which the most reliable comparisons were obtained.

TABLE I  
AVERAGE CONCENTRATION FACTORS FOR THREE IMPORTANT METHODS

METHODS	CENTRIFUGATION TIME	CONCENTRATION FACTOR	REMARKS
Centrifugation	1 hour	x25	
Alum	5 minutes	x92	To be recommended for cultivation
Chloroform	5 minutes	x102	To be recommended for microscopic work

On the basis of simplicity and effective concentration, the alum and chloroform methods were superior to all others. There was no significant difference in their collecting ability from "synthetic" tuberculous fluids free of clumps of bacilli. In the meantime, however, a study of the specific problems encountered in spinal fluids from tuberculous meningitis indicated that each procedure may serve a special purpose, depending on whether diagnosis is to be carried out by microscopic methods or by cultivation and animal inoculation.

The great frequency with which clumps occurred in clinical specimens is illustrated in Table II, which shows detailed records of the distribution of bacilli collected by flocculation methods from four of the spinal fluids from tuberculous meningitis. These examples reveal that more than 200 bacilli were seen in clumps while only 13 were encountered as individual bacilli. Similar observations have been made by other workers; for example, Boyd<sup>6</sup> states that "... the bacilli are gathered into little clumps as in the urine. . . ."

TABLE II  
THE FREQUENCY OF BACILLARY CLUMPS IN SPINAL FLUIDS FROM TUBERCULOUS MENINGITIS AND THE POTENTIAL ADVANTAGE OF DISTRIBUTING THESE BACILLI

FIELDS POSITIVE	NUMBER OF CLUMPS AND SINGLE BACILLI IN EACH POSITIVE FIELD*	BACILLI IN CLUMPS	BACILLI SINGLE	POTENTIALLY† POSITIVE FIELDS
2/108	c8, c14	22	0	1 in 5
5/170	1s, 1s, 1s, 1s, c>50	>50	4	1 in 3
7/60	c8, 1s, 2s, c3 and 3s, c5, 2s, c12	28	8	1 in 2
3/20	1s, c4, c>100	>105	1	100 per cent

\*c8 = clump of 8 bacilli; 3s = 3 single bacilli.

†In clinical specimens calculations of the potential incidence of positive fields do not prove that each bacillus liberated from a clump will produce a positive field. Nevertheless, the figures reveal that much could be gained by distributing the bacilli which so frequently occur in clumps.

Incidental observations on the clinical samples also suggested that chloroform concentrates contained fewer bacilli as clumps than did centrifuge or alum sediments. Since the alum and the chloroform methods were approximately

equal in their ability to collect unit masses of bacilli, their action on clumped suspensions was compared. "Synthetic" tuberculous spinal fluids were prepared from bacillary suspensions which, when examined microscopically as hanging drops, revealed frequent small clumps. The tubes containing the alum precipitation samples were also shaken for ten minutes according to the routine method for the chloroform procedure. The concentrates prepared by each method were divided into two parts. One was smeared directly and the other was subjected to grinding for three minutes before being smeared. It was observed that during the shaking period the chloroform was entirely taken up in the sample. An additional 0.05 c.c. was added before recovery of a chloroform concentrate was possible.

TABLE III

COMPARISON OF THE CLUMP-BREAKING ABILITY OF ALUM AND CHLOROFORM, AS MODIFIED BY MECHANICAL GRINDING OF THE CONCENTRATES

METHOD	GRINDING FOR THREE MINUTES	POSITIVE FIELDS	BACILLI IN CLUMPS	BACILLI SINGLE	TOTAL BACILLI
Alum	-	6/20	65	6	71
	+	8/20	39	5	44
Chloroform	-	18/20	0	61	61
	+	19/20	0	68	68

The results summarized in Table III indicate that, in spite of clumpy distribution, there was a reasonable agreement in the total number of bacilli collected. Nevertheless, the shaking and the mechanical grinding of the concentrates did not distribute the clumped bacilli collected by the alum procedure. These observations, together with the increased requirement for chloroform, indicate that this reagent itself was responsible for the disruption of the clumps. Pottenger's earlier conclusions<sup>7</sup> concerning the action of lipid solvents on clumps of bacilli in clinical specimens were amply confirmed.

The examination of the spinal fluids from tuberculous meningitis was carried out whenever material was available during the course of the study. Consequently, the flocculation and lipid solvent procedures were modified several times in order to check the results of the experimental work or to improve the results in the diagnostic trials. Because of the small amounts of spinal fluid, we hoped that the results of centrifugation in the hospital laboratory could be compared with those obtained by the concentration methods we studied. Unfortunately, such a comparison was not justified on account of differences in the staining quality of the carbolfuchsin employed in the two laboratories.

Thirteen spinal fluids from 11 persons proved to have tuberculous meningitis were examined by one or more of the methods investigated. Two of these were examined by culture only and were positive. One was negative microscopically but positive on cultivation from the alum sediment. Among the 10 fluids remaining, 7 were positive by one or more of the flocculation or lipid solvent methods. Of the 3 microscopically negative specimens, 2 fluids were from persons who gave microscopically positive results in another examination (these two repeats account for the 13 fluids from 11 persons); one fluid was

obtained from a patient *in extremis* and further examination of this person was not possible. At necropsy the entire brain was found to be encased in a gelatinous exudate which covered the tubercles in the meninges.

In one spinal fluid, which was positive by direct centrifugation also, a comparison with the results obtained by the use of chloroform was interesting. Forty-three fields were examined in smears obtained by each method. In the sediment obtained by centrifugation, one positive field contained two bacilli, while in the chloroform concentrate there were 12 positive fields. The observations in these fields are presented by the system employed in Table II, as follows: 8s, 3s, 1s, 1s, 1s, 1s, c6, 1s, 1s, 3s, 1s, 1s, or a score of 23 bacillary units in 43 fields.

It must be emphasized that, although the observations in the "synthetic" fluids were supported by the results in the clinical materials, the latter did not provide an adequate means of assessing the relative value of collection methods. The amounts of material were too small for testing all the methods, and the bacillary content was too low and variable to permit significant comparisons. For the present, the experimental work constitutes the chief justification of this report.

#### DISCUSSION

Although the clinical specimens were too few to justify further comment on practical results, the work with alum and chloroform in the experimental trials showed that these procedures consistently collected more bacilli than Cheer's alcohol method or direct centrifugation; the average *concentration factors* were approximately four times higher.

The alum and the chloroform methods collected unit masses of bacilli equally well, but the chloroform was shown to be definitely superior in disrupting the clumps which occur so frequently. This method produces more positive fields and is suggested for microscopic diagnosis. The procedure recommended for qualitative purposes is as follows:

#### *Chloroform Method for Microscopic Diagnosis*

1. To each 2 c.c. of spinal fluid, add 0.05 c.c. (or 4 drops) of chloroform and shake violently for ten minutes. See special instructions for formalinized fluids.
2. Centrifuge at top speed for five minutes; discard supernatant fluid.
3. Smear entire sediment without added fixative, heat fix, stain with carbol-fuchsin, and decolorize. Use methylene blue or picric acid counterstain according to the density of the smears.

The passage of acid-fast bacteria from water to oils and lipid solvents is well established. References and recent information appear in a publication by Reed and Rice.<sup>8</sup> Ligroine,<sup>9</sup> xylol,<sup>10</sup> and chloroform<sup>11</sup> have been used in concentration methods for sputum. Pottenger's data<sup>7</sup> on the resolution of bacillary clumps by lipid solvents were confirmed in our experience with chloroform.

Inasmuch as the presence of clumps is perhaps favorable to cultivation or animal inoculation, and since practically the same number of bacilli *in toto* are collected by alum and by chloroform, it was concluded that the alum concentrates should be chosen for cultivation or animal inoculation. The recommended procedure follows:

*Alum Flocculation Method for Cultivation or Animal Inoculation*

1. Pipette the specimen forcibly into an autoclaved 1 per cent aqueous solution of potassium alum\* (0.25 c.c. alum per 2 c.c. of fluid). Shake immediately for ten minutes.

2. Centrifuge for five minutes; discard supernatant fluid.

3. Cultivate the sediment on 3 to 5 tubes of suitable medium or emulsify and inject into guinea pigs.

For microscopic purposes the alum concentrates are superior to sediments obtained by direct centrifuging for one hour. They should be smeared in a very small drop of the alum-milk fixative and stained as previously indicated.

#### SUMMARY AND CONCLUSIONS

1. A study of the bacteriologic diagnosis of tuberculous meningitis by means of chemical flocculation and by lipid solvent methods showed that alum and chloroform provided more effective concentration of the bacilli during five minutes' centrifugation than was obtained by direct centrifugation for one hour.

2. For microscopic diagnosis it was important to distribute the clumped bacilli as well as to obtain efficient concentration; for this purpose the chloroform procedure was superior.

3. The alum flocculation method is recommended for cultivation or guinea pig inoculation.

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\*A film is formed on the tube in which the alum is autoclaved, but the effectiveness of the solution is not impaired.

# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**TYPHOID FEVER, Intracellular Bacilli in Intestinal and Mesenteric Lesions of,** Adams, J. W., Jr. *Am. J. Path.* 15: 561, 1939.

Gram-negative, intracytoplasmic bacillary forms, judged to be *E. typhi*, have been found in the cytoplasm of young plasma cells located in the lymphoid follicles of the ileum, colon, and mesenteric lymph nodes in 5 patients with early typhoid fever.

A method for staining intracellular bacteria is described. It is concluded that the presence of these bacillary forms within the plasma cell is an essential part of the early, classical, intestinal and mesenteric lesions of typhoid fever.

Method:

1. Stain in Weigert's iron-hematoxylin solution for one minute or less.
2. Wash in 50 per cent alcohol, acidulated to 0.1 per cent hydrochloric acid.
3. Stain in Goodpasture's carbol-aniline-fuchsin solution for one minute.
4. Decolorize in a 5 per cent aqueous solution of acetic acid until the red color disappears or the sections remain a light pink.
5. Repeat steps 3 and 4 three times.
6. Wash in water.
7. Stain in a 0.01 per cent light green solution, acidulated to 0.2 per cent acetic acid, for about one minute, or until the section has a light green hue. Avoid overstaining.
8. Wash in water, differentiate in 95 per cent alcohol, dehydrate in absolute alcohol, clear in xylol, and mount in balsam.

The nuclei stain black; the nucleoli, red; cytoplasm, pale green; bacteria, brilliant red; red blood cells, red; other structures, green.

**PANCREAS, Islet Cell Tumors of,** Campbell, W. R., Graham, R. R., and Robinson, W. L. *Am. J. M. Sc.* 198: 445, 1939.

Four cases of islet cell tumor of the pancreas and one case of diffuse hyperplasia of islets with hypertrophy of islet cells are presented.

A varied clinical symptomatology, dependent on the hypoglycemia, is shown, which parallels the results of experimental hyperinsulinism in animals. From the mildest of isolated episodes, the attacks run a course eventually resulting in marked mental deterioration and degeneration of the nervous system; marked obesity occurs as the disease progresses. Thus early diagnosis and adequate treatment are especially desirable, not only to prevent the unfavorable later results, but also to avoid the increased technical difficulties encountered in later operation. While in mild diffuse hyperinsulinism dietary treatment may suffice and the patient may revert to normal, the process in tumor cases is progressive, and nothing short of excision of the tumor will give the patient relief from his symptoms. The islet cells in these tumors exceed the mass of islet cells in the normal pancreas, and their insulin content is from 4 to 40 times that of the normal pancreas per gram, but not in toto. The clinical symptoms seem largely dependent upon uncontrolled secretory activity of the tumor rather than upon continuous production of excess quantities of insulin until the later stages, when the latter factor plays a larger role.

**CL. WELCHII, Observations on a Reaction Between the Lethal Toxin of (Type A), and Human Serum,** Nagler, F. P. O. *Brit. J. Exper. Path.* 20: 473, 1939.

A reaction is described between the lethal toxin of *Cl. welchii* (type A) and the normal human serum in which the serum becomes opalescent. When the opalescent serum is centrifuged, a fatty material rises to the surface.

The reaction is specifically inhibited by *Cl. welchii* antitoxin.

The reaction can also be observed if *Cl. welchii* of any type (A, B, C, or D) is grown in a mixture of normal human serum and broth. This cultural reaction has been shown to be specific for *Cl. welchii* and specifically inhibited by *Cl. welchii* antitoxin.

The lethal toxin of *Cl. welchii* (type A) can be titrated by means of the reaction, the activity of the toxin in bringing about the change in human serum running parallel to its toxicity for mice.

*Cl. welchii* (type A) antitoxin can likewise be titrated, the inhibiting action of the antitoxin in vitro running parallel to its neutralizing action in vivo.

**Technique.**—Cultural reaction in human serum: Three-tenths cubic centimeter of a mixture of equal parts of inactivated human serum and nutrient broth is pipetted into a test tube, of approximately 5 mm. internal diameter. Three-tenths cubic centimeter of the same mixture is pipetted into a similar test tube, to the contents of which is added 1 drop of *Cl. welchii* (type A) antitoxic serum, containing at least 20 units per c.c.

Both tubes are inoculated with 1 drop of culture of *Cl. welchii* and incubated for sixteen hours in an anaerobic jar. While the great majority of strains of *Cl. welchii* grow in pure serum, a small percentage will grow only in the serum-broth mixture. The latter strains do not readily produce a stormy clot in milk, although by all other criteria, biologic and serologic, they are true *Cl. welchii*.

After incubation the tubes are examined. In the tube without antitoxin there is an obvious opalescence, with a fatty layer at the top of the medium. The bacteria are seen at the bottom of the tube, although in some mucoid strains they are suspended in the medium. If doubt exists whether an opalescence in the medium is due to the suspended bacteria or to a change in the medium, centrifugation will clear up the doubt. After centrifugation a fatty layer will be found at the top of the medium, and bacteria at the bottom of the tube. In the tube with antitoxin the medium remains clear, with a sediment of bacteria.

#### PNEUMOCOCCI, A New Method of Culturing Sputum on Solid Media Using Carbon Dioxide for the Isolation of, Auger, W. J. Brit. J. Exper. Path. 20: 439, 1939.

A "sputum plate" technique for the isolation of pneumococci is presented.

The stimulating effect of carbon dioxide on respiratory pathogens, particularly pneumococci, has been noted.

A maximum growth of pneumococci in fifteen hours on blood agar plates is possible with this method. The method follows:

The sputum is collected from the nasopharynx in children or from the larynx in adults, using the aspiration method. This latter method ensures that the patient does not salivate instead of expectorate. The sputum is then washed in a Petri dish with about 10 to 20 c.c. of saline, which tends to remove mouth organisms and leave the purulent particles containing the likely pathogenic bacteria. A fresh blood agar plate (not older than eighteen hours) is then inoculated. This culture plate consists of 14 c.c. of 2 per cent nutrient agar, to which is added 1 c.c. of human or sheep's blood. The purulent particle is well stirred in the saline and then picked out, using a straight piece of platinum wire. The size of this particle depends on the appearance of the sputum on direct smear. If there are numerous organisms present, the particle must be correspondingly small, and vice versa. The chief difficulty of the procedure lies in the selection of a suitable particle; it should be no larger than 2 mm. in diameter. If there is any doubt about the particle being representative, the procedure should be carried out in duplicate. This particle is then placed on the center of the plate and spread thinly with a glass spreader. The spreading must be done carefully; the best method is to make two or three initial spreads across the plate in one direction. The spreader is then flamed, and the margins of the initial streak are spread upon the rest of the plate. The glass spreader itself must not be too thin or it will cut the media, and it must be held tightly while stroking. When one is experienced, one-half or one-third of a plate is sufficient for one sputum culture.

A piece of wet blotting paper, about 3 by 3 cm., is then placed on the inner surface of the lid of the Petri dish. The blotting paper should be previously autoclaved and dipped into boiling water to prevent gross contamination of the plate.

The plate is then placed with the blood agar side uppermost in the bottom of an airtight container. In routine work ordinary 1,400 c.c. tin cans, with close-fitting lids, are used, but for more accurate work glass or metal anaerobic jars are needed. Carbon dioxide is then introduced into the vessel containing the Petri dish. This gas is obtained from a commercial cylinder and is led off into a water burette (300 c.c. capacity), and finally introduced into the bottom of the container by means of a rubber tube past the partially opened lid. The actual amount of carbon dioxide introduced does not matter, since it is equally effective over a range of 0.5 to 25 per cent. This means that one should place a minimum of about 100 c.c. of carbon dioxide in a 1,400 c.c. can to allow for leakage past the open lid. After the required amount of carbon dioxide has been introduced slowly into the bottom of the can, the tube is removed and the lid is sealed with adhesive tape. The container must be airtight and then incubated for fifteen hours between 35° and 37° C. Before being used again, it should be washed with warm water.

**GLUCOSE TOLERANCE, Analysis of 583 Tests, Watson, B. A. J. Endocrinology 25: 845, 1939.**

A study of 583 routine glucose tolerance tests on patients with glycosuria is presented. The incidence of glycosuria in routine urine examinations was 1.14 per cent. No sex difference was noted in the incidence of glycosuria or mean blood sugar values during the tolerance test.

About 15.5 per cent of the patients with glycosuria on routine examination will have evidence of a definitely disturbed carbohydrate metabolism, as indicated by the glucose tolerance test. In this age group the mean blood sugar values during the tolerance test of the overweight group were lower than those of the normal weight group, and the normal weight group were lower than those of the underweight group. Thus, obesity does not appear to predispose a disturbance of carbohydrate metabolism in this age group.

The question of the importance of family history as a predisposing factor to the development of a disturbed tolerance and ultimate diabetes is raised, as subjects with a family history of diabetes had no higher mean blood sugar values during the tolerance test than did those with no such history. Only 17 of the 109 subjects with a family history of diabetes had disturbed tolerance tests. It is noted that there were wide variations in individual responses to the glucose tolerance even in the so-called normal group.

The suggestion made is that routine tolerance tests on patients having glycosuria may be an aid to early diagnosis and treatment of a disturbed carbohydrate metabolism, thus preventing the onset of clinical diabetes.

**TUBERCULIN TEST in the Control of Tuberculosis, Doull, J. A. Am. Rev. Tuberc. 40: 634, 1939.**

It is a significant fact that, although there has been a tremendous decline in mortality from tuberculosis, there is evidence that the risk for household contacts has not been appreciably reduced. The data of Downes, for example, indicate approximately the same mortality rates for those exposed in household and born between the years 1868 and 1897 as for those exposed and born during the period 1904 to 1913. As the disease declines, therefore, it is apparently more and more concentrated in the immediate environment of the open case.

Surely, therefore, the field of action is becoming more sharply delimited. The population primarily at risk is, admittedly, not completely enumerated in any community. But a large proportion of these individuals are listed on health department records, being members of families of those who are known to have open tuberculosis or of those known to have died of the disease.

There is no place for diffuseness in the tuberculosis program. Nevertheless, time and money are being dissipated in many types of indirect effort. Only when a health depart-



ment has isolated in sanatoriums all its known open cases, when all active cases not in the open stage are under medical care, when a high proportion of all household contacts are undergoing periodic medical examination, including examination by x-rays, when a system of financial subsidy is arranged for needy dependents of wage earners who have been attacked—only then should any consideration be given to indirect methods not of established value, such as that under consideration today.

**TUBERCULIN REACTORS and Non-Reactors, X-ray Findings in, Douglas, B. H. Am. Rev. Tuberc. 40: 621, 1939.**

In mass surveys the chief consideration should be the finding of active cases of tuberculosis. The finding of evidence of inactive or obsolete lesions is of less importance.

The tuberculin test has long been known to be subject to certain errors due to anergic states that may develop under certain well-known conditions, such as during certain acute infectious diseases and in terminal states of tuberculosis itself.

The x-ray examination has certain very definite limitations. Small lesions may be inaccessible and not seen. Its value is directly related to the experience and ability of the interpreter.

Granting the presence of error, tuberculin has been useful in screening from the many the few who should be further examined by the more expensive x-ray examination.

The tuberculin test is rarely negative in the presence of significant active tuberculosis: 8 out of 500 persons, 7 of whom died.

As surveys are conducted, and ignoring the cost of an x-ray film, examination of every one in the group can be carried out with less inconvenience to those examined and with less missed cases than by using a preliminary tuberculin screening.

There are several types of x-ray examination being used that seek to bring down the cost factor, fluoroscopy, sensitized paper, and miniature films; all are of some merit but none of them yet sufficiently developed to entirely supplant tuberculin testing on the basis of accuracy or economy.

There is every indication that one or more of these x-ray examination methods will be developed to an acceptable point, and tuberculin will then be used as a diagnostic aid in such problems as the determination of infection in infants and very young children, and in cases of atypical lesions in adults where tubercle bacilli cannot be found.

The final word cannot be said on this problem as yet, but even though our tools are not perfect, we should not be discouraged in using them, standing ready to adopt new and better ones when they shall have been thoroughly tested.

**BLOOD VISCOSITY, With Special Reference to Capillary, Arterial (Approximate) and Venous Blood Specimens, Holbrook, A. A., and Watson, M. V. Am. J. M. Sc. 198: 750, 1939.**

The conclusions to be drawn from this study and the reports in the literature may be given briefly as follows:

1. There is no correlation between blood viscosity and color index.
2. There is direct relationship between the number of erythrocytes and blood viscosity.
3. The normal, average, blood viscosity value is about 5.
4. The blood samples taken from a finger tip in which hyperemia has been produced (so-called "arterial" blood) give as reliable results as capillary or venous specimens.
5. There is no correlation between venous pressure within normal limits and blood viscosity.
6. Comparative studies of capillary, arterial, and venous bloods indicate that this method gives entirely satisfactory results.

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## CLINICAL AND EXPERIMENTAL

### THE ROLE OF COENZYMES I AND II IN BLOOD OF PERSONS WITH PNEUMOCOCCAL PNEUMONIA\*

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IT IS known that pneumonia may precipitate an attack of acute pellagra in undernourished persons.<sup>1</sup> Very recently, it has been shown<sup>2,3</sup> that the coenzymes I and II are decreased in the blood of patients with clinical pellagra. Unpublished observations on 150 additional cases confirm this finding. The present report is concerned with a study of the concentration of coenzymes I and II in the blood of patients with acute lobar pneumonia before and after therapy.

#### METHOD

Twenty patients, admitted to the Cincinnati General Hospital with typical pneumococcal pneumonia of less than five days' duration, and a control group of 50 normal subjects of comparable age, were selected for study.

Determinations of the coenzymes I and II concentrations of the blood were made on these patients at the time of admission and before any medication was given. Each patient was then given either type specific serum or sulfapyridine, and the blood of the patients was studied before and after crisis and during convalescence. The method used to determine the concentration of these coenzymes is based upon the unusual growth requirements of *B. influenzae* for factors X (hematin) and V (pyridine nucleotides), and has been adequately described elsewhere.<sup>2</sup>

#### OBSERVATIONS

As can be seen by the distribution of the 20 cases in Fig. 1, the concentration of coenzymes I and II in the blood of 17 patients with acute lobar pneumonia

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was strikingly decreased. The concentration of the coenzymes of the blood of the remaining 3 patients was within the limits of normal, and 2 persons, not included in the series since they died without crisis, had normal coenzyme concentration per volume of blood just before death. The average growth stimulation for *B. influenzae* of the blood of these patients on admission to the hospital was effective in concentrations of one part of blood per 2,000 parts of media. The growth could be supported, however, in all blood dilutions by the addition of either coenzyme I or coenzyme II. Within twenty-four to forty-eight hours

### BLOOD COENZYMES I AND II IN PNEUMONIA

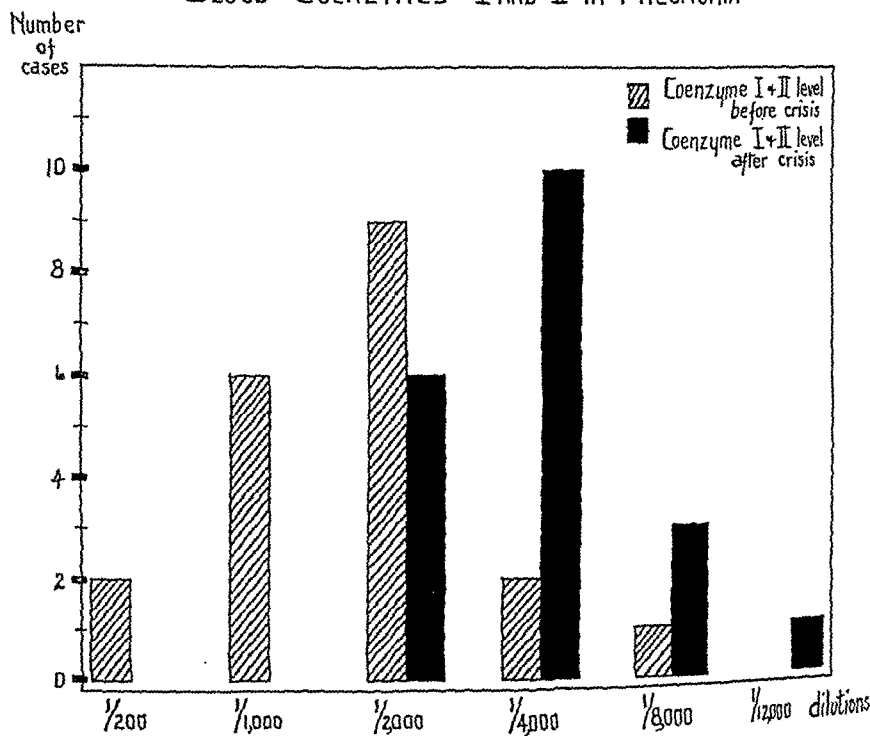


Fig. 1 illustrates the distribution of the blood coenzymes I and II concentrations in 20 persons with lobar pneumonia before and after crisis. The coenzymes I and II concentrations are measured in terms of the highest dilution of blood which supported the growth of *B. influenzae*, and are plotted against the number of cases.

after crisis induced by serum or sulfapyridine, the blood of all the patients supported growth at greater dilutions. In contrast, the average growth stimulation of the blood of the 50 normal persons for *B. influenzae* was effective at a dilution of 1:8,000. In 15 persons this dilution factor was as high as 1:12,000 and in 5, as low as 1:4,000. In the normal controls the value never fell below 1:4,000. Samples of blood taken from a normal person over a period of twenty-four hours showed little diurnal variation; likewise, samples taken from one person over a period of several months showed little deviation from the original values.

We are very grateful to Professor H. von Euler, Biochemiska Institutet, Stockholm, Sweden, for a sample of pure cozymase; and to Professor Otto Warburg, Kaiser Wilhelm Institut für Zellphysiologie, Berlin-Dahlem, Germany, for pure coferment and pure cozymase.

## SUMMARY

1. In contrast to the report of Kohn and Bernheim,<sup>4</sup> we find a significant decrease in the concentration of coenzymes I and II in the blood of 17 of 20 patients with acute lobar pneumonia, as determined by the growth stimulation for the influenza bacillus.

2. The concentration of coenzymes I and II increased to normal limits within twenty-four to forty-eight hours after a crisis induced by either serum or sulfapyridine.

3. These findings are consistent with the clinical knowledge that pneumonia predisposes malnourished persons to attacks of pellagra.

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## WATER CONTENT OF THE MYOCARDIUM IN HYPERTROPHY AND CHRONIC CONGESTIVE FAILURE\*

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IT HAS been substantiated that the counterpart of the clinical phases of cardiac hypertrophy and insufficiency lies in metabolic disturbances manifested by measurable alterations in the chemical constituents of heart muscle. In fact, it may be said that disturbances of function precede histologic changes. The anatomic alterations are effects rather than primary causes of disturbed function. In the metabolic alterations it is probable that changes occur in water content of the normal myocardium and also of the heart muscle both with and without congestive heart failure.

The purpose of this study was to determine the normal range of myocardial water content and to compare the findings with hypertrophied hearts and hearts in failure. By this means it is possible to state whether cardiac hypertrophy is due to an increase in water and whether the increase in water in the myocardium from hearts in failure is part of a generalized anasarca. Hypertrophy was deemed to be present when the heart weighed 400 or more grams; if less, there was generalized hypertrophy. Failure was regarded as present when there was frank evidence of fluid retention or chronic passive congestion of the viscera.

\*From the Medical and Laboratory Divisions of the Montefiore Hospital for Chronic Diseases.

Magnus-Levy<sup>1</sup> gives the water content of muscle as 72.24 per cent and of heart muscle as 74.81 per cent. Von Moraczewski<sup>2</sup> states that the dry substance in normal heart is 36 per cent, and that there are marked variations of water content in different conditions, with 16.9 per cent in pneumonia, 13.7 per cent in cancer, and 21.9 per cent in cancer complicated by anemia. The myocardium of the male normally shows a somewhat higher content of water than the female. The range in his material is, however, so great that the results must be doubted. Schmey<sup>3</sup> found the dry weight of fresh heart muscle from various species of animals ranging from 21.7 to 27.11 per cent. The dry weight of fresh muscle from various animals, according to J. Katz,<sup>4</sup> varies from 18.38 to 36.1 per cent. Nencki<sup>5</sup> reported that the water content of dog muscle varies between 71.7 and 74.6 per cent. Engels<sup>6</sup> found the range of water content of muscle from 11.25 to 75.36 per cent, while Wahlgren<sup>7</sup> found a variation from 71.73 to 76.11 per cent. He found that after infusions of hypertonic sodium chloride, the water content of muscle diminished, and that of the blood increased.

Hammarsten and Hedin<sup>8</sup> state that the quantity of fat has a specific influence on the quantity of fluid and that, as a rule, flesh which is deficient in water is correspondingly rich in fat. In young animals the organs in general and the muscles also have a different water content, and the uninterruptedly active heart is the richest muscle in water. Muscles of different animals have wide variations of water, and cold-blooded animals have a larger amount of water. Skelton<sup>9</sup> computed that in an average heart weighing 305.5 Gm., 241.9 Gm. are water, and that 0.58 per cent of the entire body water is in the heart. Daniels and Burright<sup>10</sup> found that the cardiac hypertrophy of rats rendered anemic by bleeding was due to an increase in muscle mass and not solely to retained water.

#### METHOD

The water content of a group of known cases of cardiac enlargement and a number with chronic congestive heart failure was compared with a control group of hearts of normal size. The tissue was obtained as shortly after death as possible, and tissue was rarely taken more than twenty-four hours postmortem. Before the heart was moistened, a sample of myocardium was regularly cut from the posterior mitral region of the left ventricle. The tissue was very lightly touched between dry cloth to remove the surrounding blood, excess pressure being avoided. The specimen was then minced in a previously weighed porcelain dish, reweighed, and put in an electric oven which was kept at uniform temperature at 56° C. by a thermocouple to prevent burning or charring. The dried muscle was reweighed several times a week until the difference between at least three successive weighings did not exceed 1 mg. Usually within three weeks uniform weight was attained. It was necessary to weigh quickly, for in several minutes the fresh specimen would give up 4 or 5 mg. of moisture, or the desiccated one would take up an equivalent amount. After several weighings it was found that if weights amounting to 2 or 3 mg. less than the previous determination were put on the pan, rapid and accurate weighing was facilitated.

#### ANALYSIS OF MATERIAL

There were 5 control patients (Table I) obtained as a result of accidental death in 4 and suicide in one. In the latter the number of hours elapsed be-

tween death and the time the specimen was obtained could not be stated, but in the other 4 the heart muscle was obtained from ten to fifteen and one-half hours after death. There were 4 males and one female. The ages varied from 8 to 47; the per cent of myocardial water was 78.4 in two, 78.6 in one, and 79.1 in a fourth. In the fifth, a child of eight years, the water content, which in childhood may normally be expected to be high, was 80.4 per cent.

TABLE I  
CONTROL GROUP

SEX	AGE	HOURS POST MORTEM SPECIMEN EXAMINED	PER CENT WATER IN MYOCARDIUM	HEART SIZE
M	47	Unknown	78.4	Normal size
F	42	14	78.4	Normal size
M	47	10	78.6	Normal size
M	47	10½	79.1	Normal size
M	8	15½	80.4	Normal size

There were 8 persons (Table II) in whom cardiac disease was a coincidental finding; 6 were females, 2 were males. Ages varied from 43 to 65 years. Heart muscle specimens were obtained from four to twenty-four hours post mortem. Two had carcinomas; one an adenocarcinoma of the rectosigmoid region with intestinal obstruction and bilateral hydronephrosis and rheumatic mitral stenosis and insufficiency with cardiac hypertrophy; the other a carcinoma of the tongue with metastasis and syphilitic aortitis with aneurysm. The heart weight of the first was 400 Gm., of the second 210 Gm. There were no cardiac symptoms during life. Water content of the myocardium in the first was 78.9 per cent, and in the second 77.6 per cent. The third had systolic and diastolic hypertension, with multiple cerebral attacks and coronary sclerosis. At necropsy both sides of the heart were enlarged; the heart weighed 450 Gm and the water content was 79.3 per cent. One with chronic pulmonary tuberculosis and cavitation, diabetes mellitus, and an enlarged liver, showed the right ventricle to be hypertrophied and dilated. The heart weight was 350 Gm., and the water content of the myocardium was 75.2 per cent. There was no congestive failure in any. No relationship existed between the degree of cardiac hypertrophy and the water content. The relatively low percentage of 75.2 water in the heart of a tuberculous patient may be explained on the basis of dehydration in a chronic wasting disease.

Eight persons, 5 females and 3 males (Table III), had renal insufficiency in addition to cardiac disease which terminated in uremia. Four had chronic glomerulonephritis, 3 had arteriosclerosis of the kidneys, and one had renal tuberculosis. Ages varied from 18 to 60 years. Specimens were obtained from three to eighteen and a half hours post mortem. Of the four with glomerulonephritis, one had associated rheumatic mitral and aortic valve disease. Heart weights varied from 385 to 710 Gm. All except one were in failure. The water content of those in failure was 79.6 in 2, and 80.7 per cent in another. The fourth had been in failure previously but not at the time of death. The water content of this patient was 78.39 per cent, the lowest of the four. Three others with hypertension and primary contracted kidneys, terminating in uremia, had congestive failure of varying duration. Heart weights were 900, 650, and 480

TABLE II  
GROUP WITH COINCIDENTAL CARDIAC INVOLVEMENT

SEX	AGE	PRIMARY DISEASE	CARDIOVASCULAR SYMPTOMS	POST-MORTEM FINDINGS	HOURS POST MORTEM SPECIMEN OBTAINED	HEART WEIGHT IN GRAMS	PER CENT WATER IN MYOCARDIUM
F	58	Adenocarcinoma of rectosigmoid, with extension to pelvis and intestinal obstruction; bilateral hydro-nephrosis and hydronephrosis	Recurrent rheumatic endocarditis, mitral stenosis and insufficiency; hypertrophy and dilatation of heart; B.P. 135/60	Mitral stenosis and insufficiency; cardiac hypertrophy and dilatation; subendocardial fibrosis left ventricle	11	400	78.9
M	50	Carcinoma of tongue, with metastasis to cervical lymph nodes; central nervous system syphilis	None; B.P. 130/70	Syphilitic aortitis with aneurysm; mild coronary sclerosis	4	210	77.6
F	56	Chronic pulmonary tuberculosis with cavitation; bronchiectasis; diabetes mellitus; hepatic enlargement	None; B.P. 100/58	Hypertrophy and dilatation of right ventricle; moderate sclerosis of left anterior descending and right circumflex coronary arteries	11	350	75.2
F	65	Hypertension; multiple cerebral infarcts; anginal attacks	None; B.P. $\frac{140-170}{100}$	Enlargement of both sides of heart; areas of subendocardial fibrosis; coronary atherosclerosis	5	450	79.3
M	54	Acute appendicitis; hypertension; obesity	Mild hypertension; appendectomy followed by terminal broncho-pneumonia	Slight cardiac hypertrophy; status postappendectomy; broncho-pneumonia	8½	410	77.2
F	48	Weakness, cough, blood-streaked sputum attributed to pulmonary tuberculosis	Cachexia; distant heart sounds; B.P. 90/62	Hypertrophy of right heart arterio-sclerosis of kidneys	11	300	79.9
F	62	Hypertension, 20 years; thrombo-phlebitis migrans, 9 years	Hypertension; phlebitis migrans; terminal cerebral insult; B.P. $\frac{220-300}{124-180}$	Renal arteriosclerosis; nephrolithiasis; cardiac hypertrophy and dilatation	6	500	79.08
F	43	Weakness, fever, cough, lupus erythematosus, hematuria and proteinuria, 3 years	Lupus erythematosus; scleroderma; atrophy of musculature; hepatosplenomegaly; B.P. 102/81	Lupus erythematosus; adherent pericardium; abscess of pericardium; indeterminate endocarditis of mitral and aortic valves	24	280	77.8

Gm., and the percentage of water 80.2, 78.57, and 78.2, respectively. A patient with extensive renal tuberculosis, hypertension, and cerebral insult terminated also in uremia. There was no history of congestive heart failure. The heart weight was 460 Gm., but the water content of the myocardium was 77.9 per cent. It is noteworthy that the water content of the myocardium of the persons in whom there was no failure was low, and that the lowest water content in the entire group was in a person not in failure. The highest percentage of myocardial water in the entire group, 80.7, was from a patient with generalized anasarca.

Congestive heart failure, which had been present for ten years in two persons, cleared up before death (Table IV). Both showed cardiac hypertrophy, with heart weights of 540 and 500 Gm. and both had extensive coronary artery disease; one had, in addition, rheumatic heart disease, and the other had a large patent foramen ovale. The water content of the myocardium of the first was 79.3 and of the second, 79.7 per cent. Failure was, however, absent, and water content was considerably less than in many persons with congestive heart failure.

In addition to the 6 persons whose condition was complicated by nephritis, 32 other persons had congestive heart failure at the time of death (Table V). Fifteen were males, 17 were females. Ages varied between 13 and 75 years. Material was obtained from one and a half to twenty-seven hours post mortem, but in the majority of instances the specimen was obtained within twelve hours after death. Of these 11 had rheumatic heart disease. Every one also had cardiac hypertrophy. Heart weights varied from 350 to 730 Gm. The percentage of water in the myocardium varied between 78.9 and 82.1. The latter figure was the highest in the entire series. It is interesting that 7 of the 11 had myocardial water in excess of 80 per cent. There was no relationship between the degree of cardiac hypertrophy and the amount of water present in the myocardium. The high percentage of water in this group may be explained by the fact that many hearts were from children whose hearts normally have a high water content.

The presence of hypertension or a history of it was noted in 17. Cardiac hypertrophy and dilatation were present in all. The average heart weight was 521 Gm., approximating the figures usually given for hypertensive heart failure. The lowest figures for water were 77.3, 78.03, and 78.7 per cent. The first of these was from a hypertensive patient with chronic pulmonary tuberculosis, a colostomy for cancer of the rectum, and cirrhosis of the liver. Heart weight was 380 Gm. and hypertrophy was limited to the right side of the heart. The low water content may be explained by associated chronic wasting disease and by advanced age. Figures in excess of 80 per cent occurred less frequently than in the rheumatic group since the patients were adults with essential hypertension; however, significant increases in water occurred in many.

A child with patent ductus arteriosus, subacute bacterial endocarditis, acute glomerulonephritis, and congestive failure, had a heart weighing 275 Gm., whose water content was 81.4 per cent. A woman of 64 years with Grave's disease, cardiac hypertrophy, and congestive failure had a heart which contained 79.2 per cent water.



TABLE III  
GROUP COMPLICATED BY NEPHRITIS

SEX	AGE	PRIMARY DISEASE	CARDIOVASCULAR SYMPTOMS	POST-MORTEM FINDINGS	HOURS POST MORTEM SPECIMEN OBTAINED	HEART WEIGHT IN GRAMS	PER CENT WATER IN MYOCARDIUM
F	60	Chronic nephritis; diabetes mellitus; hypertension; coronary artery disease, $3\frac{1}{2}$ years before admission; unconsciousness; coronary occlusion followed by swelling of legs; left ventricular failure	Cardiac enlargement; uremia; marked secondary anemia; hypoproteinemia; hiatus diaphragmatic hernia; paroxysmal nocturnal dyspnea and basal rales; uremic pericarditis; B.P. 220/120	Chronic glomerulonephritis; cardiac hypertrophy uremic pericarditis, not in congestive failure post mortem	3	385	78.39
M	18	Colds and chronic sinusitis followed by nephritis	Hematuria; hypoproteinemia; anemia; hypertension; low blood calcium; high blood phosphorus; B.P. 170-180 B.P. 110-120; nephritic acidosis; pulmonary congestion pericarditis and congestive heart failure 6 weeks before death	Chronic glomerulonephritis; pericarditis; pleuritis; colitis; chronic passive congestion of viscera; anasarca	16	770	79.6
M	44	Cold followed by headache, swelling of eyelids and ankles $1\frac{1}{2}$ years before admission; hypertension and diabetes mellitus, $1\frac{1}{2}$ years; dyspnea on exertion, 10 months; congestive heart failure, 6 months	Cyanosis, dyspnea, orthopnea; impaired vision; hypertensive retinopathy; peripheral edema and paroxysmal dyspnea; progressive failure and anasarca; uremia; B.P. 220/140	Chronic glomerulonephritis; hypertrophy and dilatation of heart; chronic passive congestion of viscera; anasarca	10	410	80.7

M	45	Hypertension and dyspnea on exertion, 3 years; congestive failure, 1 year; acute coronary closure, 4 weeks before admission	Dyspnea, sweating, precordial distress; systolic apical murmur; hepatic enlargement; hypertensive encephalopathy; B.P. 95-110; uremia; auricular fibrillation	Fresh occlusion, left anterior descending; partial occlusion right transverse; infarction septum, apex, anterior and posterior walls of left ventricle; chronic passive congestion of viscera; arteriolosclerosis of kidneys	19	900	80.2
M	44	Acute rheumatic fever at 19; dyspnea, orthopnea; ankle edema; enlargement of abdomen, 3 years; syphilis	Blood Wassermann 1-2 plus; orthopnea; cyanosis; pulmonary congestion; hepatic enlargement; auricular fibrillation; B.P. 180/90; congestive heart failure practically cleared up	Healed rheumatic endocarditis of mitral and aortic valves; pulmonary infarct; chronic passive congestion of viscera; hydrothorax; ascites; chronic glomerulonephritis	18.5	400	79.6
F	50	Diabetes, 11 years; hypertension, 9 years; congestive failure, 4 years	Congestive failure; auricular fibrillation; peripheral edema; hepatic enlargement; ascites; B.P. 175/100	Hypertrophy and dilatation of heart; arteriolosclerosis of kidneys; uremic colitis; chronic passive congestion of viscera	12	650	78.57
F	60	Progressive congestive failure, 8 years; abdominal paracentesis repeatedly done	Auricular fibrillation; ascites, terminal uremia; B.P. 120/80	Cardiac hypertrophy and dilatation; coronary and aortic sclerosis; renal arteriolosclerosis; chronic passive congestion of viscera; bronchopneumonia	Not stated	480	78.2
F	40	Renal tuberculosis, 15 years; hypertension, 6 years; right hemiplegia, 1 year; anginal pain, 3 years	Renal insufficiency, hypertension; right hemiplegia; B.P. 240/150	Tuberculosis of kidneys with extensive excavation; hypertrophy of heart; bronchopneumonia	4	460	77.9

TABLE IV  
HYPERTENSIVE PERSONS IN WHOM CONGESTIVE HEART FAILURE CLEARED UP BEFORE DEATH

SEX	AGE	PRIMARY DISEASE	CARDIOVASCULAR SYMPTOMS	POST-MORTEM FINDINGS	HOURS POST-MORTEM SPECIMEN OBTAINED	HEART WEIGHT IN GRAMS	PER CENT WATER IN MYOCARDIUM
M	68	Hypertension; diabetes mellitus, 10 years; amputation of leg; dyspnea; orthopnea; paroxysmal nocturnal dyspnea, 6 months	Auricular fibrillation; liver 1 finger below costal margin; not in failure at time of death	Marked sclerosis of right and left coronary arteries; recent infarction of apex; rheumatic mitral and aortic stenosis and insufficiency	17	540	79.3
M	60	Precordial pain; palpitation and dyspnea 10 years before admission, followed by congestive heart failure	Emaciation, cyanosis, orthopnea; pulmonary congestion; auricular fibrillation; hepatic enlargement; pretibial edema; congestive heart failure cleared up; anginal seizure followed by death in 6 hours	Heart weight 500 Gm.; recent occlusion of left coronary artery; scarring of left ventricle and right auricle; large patent foramen ovale	9	500	79.7

There were 2 persons with advanced pulmonary tuberculosis with advanced congestive heart failure. The heart of one weighed 380 Gm. and showed hypertrophy and dilatation of the right side. The water content was 81.5 per cent. The heart of the other weighed 560 Gm., had failure of the right side of the heart of one and a half years' duration, and a water content of 79.69 per cent. The marked increase in muscle mass in the second person may have been due to some factor other than lesser circulation hypertension.

#### DISCUSSION

The water content of all the tissues of the body varies at different times and under different circumstances. It is higher in youth, declines in maturity, and increases in old age. Different species of animals have different amounts of water in the tissues. The most active organs of the body have the greatest percentage of water. The brain, the heart, and the muscles accordingly have the highest water content. Various diseases, wasting states, dehydration, and hydremia may be expected to alter the water content of organs.

Peters and Van Slyke<sup>11</sup> states that abstinence from water causes a reduction of water content of blood, whereas an excess intake causes an increased content in the blood. Muscles do not take up fluid after injections of sodium chloride and calcium chloride. Following injections of hypertonic salt solutions, muscles even give up water, probably as a compensatory mechanism. In general, however, water content of muscle may be expected to remain at a fairly uniform level.

Since water is most abundant in the tissues in youth, the water of the myocardium in childhood is also very high. The heart of a normal child had 80.4 per cent water, the highest in the normal group. It seems reasonable also that the greatest water content of the entire series should be found in the hearts of children with congestive heart failure. This view was confirmed. The highest figure in the series, 82.1 per cent, was recorded from a child of 16 with rheumatic heart disease and generalized anasarca.

The regulation that is maintained over water balance makes it unlikely that there can be very great variations in water in the myocardium. The hearts of patients with malignancies or chronic wasting diseases, such as tuberculosis, in which congestive failure is terminal or coincidental may show the effects of chronic dehydration and inanition. Several such cases, despite advanced failure, showed an amount of myocardial water within normal limits. Age may be an additional contributory factor since water content is low in maturity. It is, therefore, reasonable to expect, and it was confirmed in this study, that in congestive heart failure in the aged, water content may be within or only slightly above the normal. When it is considered that the increase in water in the myocardium due to congestive failure is not great, it is evident that in failure complicated by wasting diseases or advanced age, normal figures may represent actual water retention.

The close guard that the kidneys maintain over the electrolyte constants of the blood makes it likely that normal limits of water will not be exceeded in compensated renal insufficiency, but will be increased when renal disease is

TABLE V  
PERSONS SHOWING CONGESTIVE HEART FAILURE

SEX	AGE	PRIMARY DISEASE	CARDIOVASCULAR SYMPTOMS	POST-MORTEM FINDINGS	HOURS POST MORTEM SPECIMEN OBTAINED	HEART WEIGHT IN GRAMS	PER CENT WATER IN MYOCARDIUM
M	66	Precordial pain; dyspnea and swelling of lower extremities of several years' duration; thyroidectomy for Graves' disease	Cyanosis of lips, dyspnea; thyroidectomy scar; acute coronary thrombosis followed by increased congestive failure; B.P. 190/100	Thrombosis of branches of left circumflex coronary artery; marked enlargement of both sides of heart	4	640	79.7
F	73	Hypertension, 10 years; thyroidectomy for Graves' disease 6 years before admission; congestive heart failure, 9 years	Thyroidectomy scar; cyanosis, dyspnea; pulmonary congestion; cardiac enlargement; B.P. 136/74	Old occlusion of left anterior descending; healed infarct of septum; subendocardial fibrosis of both ventricles; bilateral hydrothorax	12	520	80.8
M	63	Hemiplegia 12 years before and shortly before admission; dyspnea, palpitation, and precordial pain 12 years; abdominal and precordial pain 6 weeks and anasarca 5 weeks before admission	Residual right hemiplegia; precordial pain; dyspnea; congestive heart failure with anasarca; B.P. 140/70	Hypertrophy and dilatation of all chambers; marked fibrosis of lateral wall and thinning of septum; marked coronary narrowing and canalization	14	550	79.7
M	66	Coronary thrombosis 2 years before admission followed by progressive congestive heart failure, paroxysmal dyspnea, and paroxysmal auricular fibrillation	Hypertension; cardiac enlargement; congestive failure; ventricular aneurysm; pulmonary infarction; B.P. 110/76 - 140/110	Old occlusions of left anterior descending and transverse marked narrowing of right transverse coronary arteries; old infarcts of apex, septum, anterior and posterior walls of left ventricle; infarcts of lung; chronic passive congestion of viscera	6	630	78.03
F	13	Joint pains; recurrent Rheumatic endocarditis of mitral, aortic, and tricuspid valves, 2½ years; rheumatic purpura	Active carditis; mitral and aortic valvular disease; congestive heart failure; ascites B.P. 139/60	Healed and recurrent rheumatic endocarditis of mitral and aortic valves and fibrinous pericarditis; acute rheumatic myocarditis	5	520	80.6
M	54	Rheumatic heart disease; dyspnea on exertion, 4 years; edema of ankles, 1 year	Healed and recurrent rheumatic endocarditis and myocarditis; mitral stenosis and insufficiency; chronic passive congestion of viscera; hydrothorax; ascites	Healed and recurrent rheumatic endocarditis and myocarditis; mitral and aortic stenosis and insufficiency; hydrothorax; ascites	4	730	79.3

F	16	Rheumatic heart disease, 13 years; dyspnea; palpitation, precordial pain, 2 years	Rheumatic heart disease; mitral stenosis and insufficiency; aortic insufficiency; progressive congestive heart failure	Healed and recurrent rheumatic endocarditis of aortic valve; hypertrophy and dilatation of heart; pulmonary infarcts; chronic passive congestion of viscera	8	490	82.1
F	13	Patent ductus arteriosus; subacute bacterial endocarditis; cough, bronchitis, and pyrexia, 2 months	Patent ductus arteriosus; subacute bacterial endocarditis; pulmonary infarcts; pyrexia; consolidation, right base	Acute glomerular nephritis; patent ductus arteriosus; subacute bacterial endocarditis of mitral, pulmonary artery; pulmonary valves and congenital cystic disease of lungs, pulmonary infarcts	3	275	81.8
M	42	Hypertension, 4 years; swelling of legs, 6 months	Dyspnea, cyanosis, pulmonary and hepatic congestion; anasarca; cerebral insult; B.P. 134/104	Occlusion of all major coronary arteries; recent and old infarction of myocardium; thrombosis of splenic artery with infarction; cardiac cirrhosis of liver; ascites; hydrothorax	13	660	80.9
M	75	Ankle edema, 5 years; dyspnea and orthopnea, 9 months; edema of lower extremities, 9 months; edema of upper extremities, 1 month	Cyanosis; pulmonary congestion; hepatic enlargement; cardiac enlargement; thrombophlebitis; Parkinsonism; B.P. 165/105	Recent occlusion of small branch of left descending and recent infarction of apex; old infarct of anterior wall; infarct of spleen, hydrothorax	11	810	79.3
F	65	Osteoarthritis; diabetes mellitus, 7 years; adenocarcinoma of uterus, 2½ years; precordial pain and paroxysmal dyspnea	Paroxysmal dyspnea; auricular fibrillation; enlargement of liver and spleen; B.P. 140-200/85-90	Marked narrowing of major coronary arteries, recent infarction of apex and posterior wall of left ventricle, hypertrophy and dilatation of all chambers; congestion of liver and lungs	5	380	81.1
M	30	Advanced bilateral chronic pulmonary tuberculosis; pyopneumothorax and bronchopleural fistula, 4 years	Pulmonary congestion; sudden dyspnea and cyanosis 2 days before death, and acute pulmonary congestion	Hypertrophy and dilatation of right auricle and ventricle; liver markedly enlarged; central and peripheral vessels congested	Not stated	380	81.5
F	57	Hypertension; dyspnea on exertion and angina pectoris, 3 years; vomiting, 7 months; tumor in epigastrium and abdominal pain, 7 months; anorexia, constipation, loss of weight	Cachexia; poor pulse and heart sounds; epigastric mass extending to right and left and attached to liver; B.P. 136/100	Old infarcts of anterior and posterior walls of left ventricle and septum; hypertrophy and dilatation of all chambers; Graetz tumor of kidney with metastasis; congestion and edema of lungs	22	480	80.0

TABLE V—CONT'D

SEX	AGE	PRIMARY DISEASE	CARDIOVASCULAR SYMPTOMS	POST-MORTEM FINDINGS	HOURS POST MORTEM SPECIMEN OBTAINED	HEART WEIGHT IN GRAMS	PER CENT WATER IN MYOCARDIUM
F	71	Postoperative carcinoma of breast; B.P. 190/130; hemorrhage followed by dyspnea, edema of ankles, pulmonary congestion, transient auricular fibrillation, severe anemia	Dyspnea; pulmonary congestion; edema of ankles; transient auricular fibrillation	Hypertrophy of right and left ventricles; dilatation of all chambers; moderate coronary sclerosis	4½	380	79.6
F	65	Colostomy for carcinoma of rectum; chronic pulmonary tuberculosis; diabetes mellitus, 9 years; cirrhosis of liver	Episodes of cyanosis; pulmonary congestion; liver edge 4 fingers below costal margin; B.P. 200/90; fell to 110/80 following episode of dyspnea and cyanosis	Old occlusions of left anterior descending; old and recent thrombus of right transverse and recent infarction posterior wall of left ventricle; fibrosis of septum; chronic passive congestion of viscera	16	550	77.3
F	75	Hypertension and congestive heart failure, 10 years; cerebral arteriosclerosis and psychosis; fracture of femur	Cardiac enlargement; auricular fibrillation; cyanosis; orthopnea; peripheral edema; chronic bronchitis; B.P. 190/100	Coronary arteriosclerosis; chronic passive congestion of viscera; emphysema; chronic bronchitis	5	480	78.7
F	58	Cerebral insult 6 years before admission; hypertension known 8 years; precordial pain, dyspnea, and edema, 2 years; diabetes mellitus	Residual left supranuclear facial palsy; cardiac enlargement; congestive failure; mild diabetes mellitus; paroxysmal dyspnea	Cardiac hypertrophy; thrombosis of pulmonary vein; infarct of lung; chronic passive congestion of viscera	13	460	79.04
M	53	Far-advanced chronic pulmonary tuberculosis, 19 years; hypertension; progressive dyspnea on exertion, 3 years	Chronic advanced pulmonary tuberculosis; right heart failure; B.P. 150/110; amyloidosis	Healed bilateral pulmonary tuberculosis hypertrophy of both ventricles and left auricle; congestion and edema of viscera; renal arteriosclerosis	Not stated	400	79.6
M	16	Double mitral murmurs discovered at 10 years of age; several months later, fever and tachycardia; congestive heart failure, 3 years	Rheumatic mitral stenosis and insufficiency; tricuspid insufficiency; auricular fibrillation; ascites and hydrothorax; B.P. 130/70	Healed and recurrent rheumatic mitral stenosis; aortic and tricuspid insufficiency; chronic passive congestion of viscera	14	740	78.9

F	62	Dyspnea, palpitation, precordial pain, 5 years; abdominal distention	Mitral stenosis and insufficiency; auricular fibrillation; congestive heart failure; adenoma of thyroid; B.P. 145/95	Adherent pericardium; rheumatic endocarditis of mitral valve; hypertrophy and dilatation, especially of right side of heart; cardiac and Laënnec's cirrhosis; chronic passive congestion of viscera; bronchopneumonia	30	540	80.0
F	24	Heart trouble found at 6 years of age; occasional migratory joint pains and swelling; pain in abdomen and hips and red spots under nails, 8 months	Rheumatic mitral stenosis and insufficiency; cardiac enlargement; subacute bacterial endocarditis due to <i>Streptococcus viridans</i>	Rheumatic mitral stenosis and insufficiency; subacute bacterial endocarditis of mitral valve; chronic passive congestion of viscera; infarction of spleen and kidneys	3	350	81.2
F	44	Left hemiplegia, 6 years; weakness and dyspnea on exertion, 6 years; frequent attacks of palpitation; abdominal discomfort and swelling of abdomen, 2 years	Cardiac enlargement; double murmur over mitral area; hepatic enlargement; ascites; pretibial edema	Myxoma of left auricle; cardiac hypertrophy and dilatation; chronic passive congestion of viscera; ascites and bilateral hydrothorax	16	550	81.4
F	72	Cerebral insult 19 years before admission; fatigability and dyspnea on exertion, 2 years; recent enlargement of abdomen	Disorientation; cardiac enlargement; pulmonary congestion; hepatic enlargement; peripheral edema; ascites; B.P. 170/90	Infarct of left ventricle; marked coronary sclerosis; thrombus of pulmonary artery; cardiac hypertrophy and dilatation; chronic passive congestion of viscera; arteriosclerosis of kidneys	21	450	79.4
M	32	Rheumatic fever, polyarthritis and valvular lesions at 12 years; chills, fever, anorexia, pallor 5 months before death; cerebral insult with right hemiplegia	Enlarged heart; mitral stenosis and insufficiency; aortic insufficiency; hepatomegaly; hemiplegia and aphasia; subacute bacterial endocarditis with positive blood culture; B.P. 120/0	Chronic rheumatic cardiovascular disease of mitral and aortic valves; subacute bacterial endocarditis of mitral valve, left auricle and left ventricle; chronic passive congestion of viscera; acute focal hemorrhagic nephritis	11	630	79.7
M	25	Rheumatic heart disease; mitral stenosis and insufficiency; subacute bacterial endocarditis	Multiple embolic phenomena, pyrexia of long duration; hepatosplenomegaly; developed terminal congestive heart failure	Chronic rheumatic mitral and aortic insufficiency; superimposed subacute bacterial endocarditis; chronic passive congestion of viscera; bilateral hydrothorax	3	500	80.52



TABLE V—CONT'D

SEX	AGE	PRIMARY DISEASE	CARDIOVASCULAR SYMPTOMS	POST-MORTEM FINDINGS	HOURS POST MORTEM SPECIMEN OBTAINED	HEART WEIGHT IN GRAMS	PER CENT WATER IN MYOCARDIUM
F	25	Pain in ankles at 8 years; double murmur found at 15 years; fever, joint pains, chills, petechial lesions, 7 months	Mitral stenosis and insufficiency; aortic insufficiency; cardiac enlargement; petechial spots; post-mortem blood culture, atypical hemolytic streptococcus; B.P. 118/55; terminal cerebral insult	Rheumatic mitral stenosis and insufficiency; cardiac hypertrophy and dilatation; subacute bacterial endocarditis; focal glomerulonephritis; mycotic infarcts of spleen and kidneys; chronic passive congestion of viscera	3	450	79.7
M	51	Dyspnea on exertion, 1½ years; palpitation, increasing dyspnea, paroxysmal nocturnal dyspnea and ankle edema, 2 months	Cardiac enlargement; marked hepatic enlargement; B.P. 134/80-110	Occlusion left anterior descending coronary artery; infarction of left ventricle; chronic passive congestion of viscera; right hydrothorax and ascites	6	670	81.9
M	11	Frölich's syndrome; rheumatic fever at 3 and 6 years; chorea at 7 years	Mitral stenosis and insufficiency; aortic insufficiency; cardiac enlargement; active endocarditis; congestive failure; Frölich's syndrome	Rheumatic mitral stenosis and insufficiency; aortic insufficiency; adherent pericardium; chronic passive congestion of viscera	17	650	80.26
F	64	Dyspnea, precordial heaviness and weakness, 1 week; congestive heart failure, 1½ years	Dyspnea, cyanosis; cardiac enlargement and irregularity; hepatic enlargement; peripheral edema; thyroid enlarged; progressive congestive failure	Adenomas of thyroid; cardiac hypertrophy and dilatation; chronic passive congestion of viscera	20½	350	79.2
F	48	Hypertension, headaches, dizziness and tremors, 8 years; hyperthyroidism; memory and personality changes, 1 year	Enlarged heart; congestive failure; elevated basal metabolism; glycosuria; terminal cerebral insult; B.P. 210/148	Cardiac hypertrophy and dilatation; pheochromocytoma; chronic passive congestion of liver; hydrothorax	11	320	79.4
M	50	Cough, expectoration, weakness, backache, 2½ years; right side heart failure, 1½ years; episodes of fever, weakness; loss of weight	Cyanosis; sibilant râles all over chest; cardiac enlargement; tachycardia; peripheral edema; B.P. 122/80	Bronchiectasis; fibrinous pericarditis; hypertrophy and dilatation of right side of heart; chronic passive congestion of viscera; ascites	27	560	79.69
M	57	Cerebral insult 10 years before admission; hypertension; diabetes mellitus, 1½ years	Systolic apical murmur; lower thigh amputation; B.P. 170/100	Amputation at thigh; marked coronary arteriosclerosis; cardiac hypertrophy; myocardial infarction; chronic passive congestion of viscera; bronchopneumonia	24	470	78.7

complicated by congestive heart failure. In 4 patients with uremia with congestive failure the water content was significantly increased; it is significant that 2 patients in uremia with hypertension and cardiac hypertrophy but no failure showed a normal amount of water in the myocardium.

In the hypertrophied compensated heart the weight of the heart had no relation to its water content. The amount of water in the heart not in failure was within normal limits. In congestive heart failure, however, the amount of water in the myocardium was definitely increased in many instances. It may be said, then, that cardiac hypertrophy is not wholly or partly due to increased water but to an increase in intrinsic muscle mass. Congestive heart failure, which causes a hydremia and edema of all the tissues, affects the heart muscle as well and is manifested by an increase in the percentage of water.

The clinical importance of the demonstration of increased water in the myocardium in congestive heart failure is of significance in connection with diuretic therapy. In addition to a primary action on the kidneys, it is probable that diuresis may exert an important secondary action on the heart muscle, resulting in reduction of edema of the heart, thereby augmenting cardiac contractility.

#### SUMMARY

1. The water content of the myocardium from persons with cardiac hypertrophy and with cardiac hypertrophy with congestive heart failure was determined and compared with normals used as controls

2. The hearts from patients with wasting diseases and the aged showed relatively low water contents. Children, on the other hand, showed relatively high myocardial water contents normally and in congestive heart failure.

3. In cardiac hypertrophy without failure the water content was not increased. The increase in heart weight in cardiac hypertrophy is due to an intrinsic increase in muscle mass and not to an increased amount of water.

4. In congestive heart failure the water content of the myocardium was increased due to anasarca which involved also the myocardium. The greatest increase of water was observed in childhood, reaching 82.1 per cent in one instance. Normal figures in the aged patients with wasting diseases may represent actual water retention.

5. The beneficial effects of diuresis in congestive heart failure may in part be due to reduction of excessive myocardial water content, thereby improving cardiac contractility.

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## DIARRHEA CAUSED BY DIENTAMOEBA FRAGILIS\*

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CLINICIANS are familiar with the patient who presents symptoms of persistent diarrhea, characterized by frequent mushy stools and associated with a certain amount of abdominal distress. Symptomatic treatment may alleviate the condition, but the original status returns when normal living is resumed. Although this type of diarrhea does not progress to a true dysentery, it is nevertheless annoying. Laboratory reports frequently show no abnormal findings either in the bacteriologic or parasitologic fields.

Such cases present a special problem to the technician. Sometimes direct saline smears of the fecal material show small, round, refractile bodies that cannot be found in either the iodine preparations or the concentration suspensions commonly used for aid in identification of intestinal parasites. Because of this, it is probable that these bodies, which may be the true etiologic factor, are ignored or classed in the general group with the vegetable organism, *Blastocystis hominis*.

### LITERATURE REVIEW

In the original article describing *Dientamoeba fragilis* Jepps and Dobell 1918<sup>1</sup> reported finding 7 cases. They believed this to be a rare form, although they pointed out that its recognition was too recent for the extent of its occurrence to be known. Credit was given to Wenyon who had seen this organism in 1909 but, not recognizing it as a new species, had not reported it.

In 1924 Taliaferro and Becker<sup>2</sup> reviewed the literature dealing with the reported cases of *Dientamoeba fragilis*. There had been 20 cases reported from England, 5 from the Philippines, 4 from Germany, 3 from the United States and one from Holland, making a total of 33 cases. Even this does not represent the wide distribution of the infection, as many of these individuals were soldiers or travelers returning from various other countries.

In 1926 Kudo<sup>3</sup> made a careful study of these organisms from a patient in Urbana, Ill. The following year Gittings and Waltz<sup>4</sup> reported finding 3 cases in children. Previously the infection had apparently been found only in adults.

Up to the year 1934 *Dientamoeba fragilis* had been considered a rare organism of widespread distribution. At this time Svensson and Linders<sup>5</sup> made a

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thorough survey of 74 persons in a heavily infected group of patients in a mental hospital in Sweden. They found 72.9 per cent of this group positive for *Dientamoeba fragilis*, and 54.0 per cent harboring *Endamoeba histolytica* organisms. The following year Wenrich, Stabler, and Arnett<sup>6</sup> made an intestinal protozoal survey of 1,060 freshmen entering the University of Pennsylvania. In this group 45, or 4.3 per cent, were found positive for *Dientamoeba fragilis* and 43, or 4.1 per cent, positive for *Endamoeba histolytica*. The next report came from Brug<sup>7</sup> who carried out his work in a mental hospital in Holland. He found 36 per cent of the patients infected with *Dientamoeba fragilis* as compared to an 8 per cent positive finding for *Endamoeba histolytica*. In 1937 Hakansson<sup>8</sup> found 16 new cases of *Dientamoeba fragilis* among 31 institutional patients in Panama, giving a 42.1 percentage of new infections. This group had previously been treated for *Endamoeba histolytica* and subsequent examinations had shown them to be free from amoebic infections.

Sapero and Johnson<sup>14</sup> reported a series of examinations made on Navy recruits at Norfolk, Va. They found 17.1 per cent of the men from the southern states and 10.4 per cent of the men from the north infected with *Dientamoeba fragilis*. This is the highest percentage ever reported for this parasite in the United States. This same group showed 14.7 per cent and 7.8 per cent *Endamoeba histolytica* infection, respectively.

#### TECHNIQUE OF EXAMINATION

Over 2,000 fecal examinations have been made in this laboratory during the last few years. Up to September, 1938, no *Dientamoeba fragilis* were identified. The reports indicate that these organisms were present but were being overlooked.

During the summer of 1938, while working at the Gorgas Memorial Laboratory in Panama, I became familiar with the *Dientamoeba fragilis* and the technique of its identification. Hakansson,<sup>8</sup> whose work was done at the Gorgas Laboratory, gave a description similar to the following. In a saline emulsion *Dientamoeba fragilis* organisms appear as small (5 to 20 microns), fine, refractile, very round bodies. When pseudopods are observed, they are very thin and show a characteristic angular appearance. Careful watching is required to observe this motion as the action is usually delayed. The nuclei are very indistinct or invisible. In water solutions, therefore, in iodine preparations frequently used to bring out nuclear structure, the body explodes, freeing the endoplasm and leaving a ring of ectoplasm which is not characteristic of the original structure but more nearly resembles a disintegrating blastocystis. This description follows very closely that given by Jepps and Dobell<sup>1</sup> in their original article on *Dientamoeba fragilis*.

For confirmation of the diagnosis iron hematoxylin stains are most useful. Such studies made by Jepps and Dobell,<sup>1</sup> Robertson,<sup>9</sup> Kudo,<sup>3</sup> Craig,<sup>10</sup> and Wenrich<sup>11</sup> have given an adequate knowledge of the nuclear structure of this organism. About 80 per cent of the forms have two nuclei of similar structure which may be adjacent or at opposite sides of the cytoplasm. The nuclear membrane is so delicate it appears as merely the termination of the cell cytoplasm.

The karyosome is made of four or more fairly distinct granules, one of which is often slightly larger and may take a somewhat darker stain than the others.

The special modification of adding an extra amount of glacial acetic acid (total 15 per cent) to the Schaudinn solution to bring out the nuclear structure of *Dientamoeba fragilis*, as suggested by Johnson,<sup>12</sup> has proved very satisfactory. This Johnson method was used for all the hematoxylin stains.

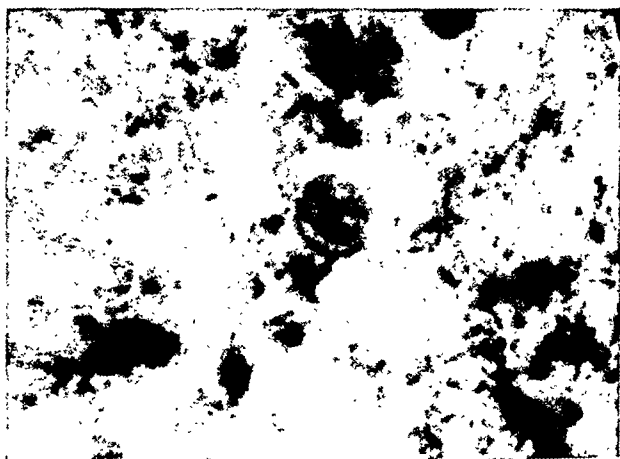


Fig. 1.

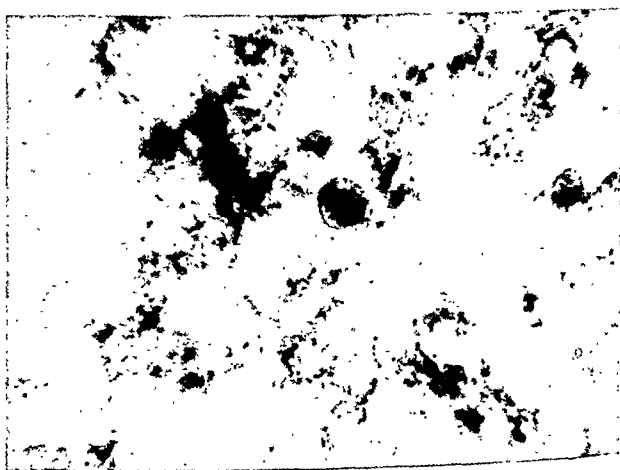


Fig. 2.

#### RESULTS

During the last ten months fecal specimens from 164 individuals have been examined. The special characteristics of *Dientamoeba fragilis* have been kept in mind. Iron hematoxylin stains were made if the direct smear showed the presence of any protozoa, and each slide was searched carefully for any form which might be present. *Dientamoeba fragilis* organisms were found in the stools of 7 patients, 3 children and 4 adults giving a 4.2 per cent positive. In this group 6 cases of *Endamoeba histolytica* were found. The relationships between the per cent positive for *Endamoeba histolytica* and *Dientamoeba fra-*

*gilis* compare favorably with the results obtained by Wenrich, Stabler, and Arnett.<sup>6</sup> The actual percentages, however, cannot be compared, as Wenrich and others were conducting a student survey, while the present work was carried out on patients whose clinical symptoms indicated the advisability of a fecal examination.

#### PATHOGENICITY

In the original article on *Dientamoeba fragilis* Jepps and Dobell stated that there was no reason to believe that this organism was a pathogen. All their cases showed some type of abdominal distress, but as few symptomless patients were sent to their laboratory, they considered this a coincidence. Mollari and Anzulovic<sup>13</sup> in 1938, quoting Robertson, Gittings and Waltz, Hakansson and adding their own case, showed that the observations of these workers pointed to the fact that *Dientamoeba fragilis* infection produces gastrointestinal disturbance and an eosinophile response in the blood.

The present small series gives further evidence of a pathogenic role for *Dientamoeba fragilis*. One of the children who also had an accompanying *Endamoeba histolytica* infection had definite intestinal distress and diarrhea. A course of treatment with chiniofon cleared up the condition. Another child who had mild diarrhea showed an 11 per cent eosinophilia. No other cause for these reactions could be elicited. This was the only one of the 7 cases that showed an increase in the eosinophile count. The mother of the third child reported finding worms in the stool and requested an examination. No symptoms had been noted. These last two children had been hospitalized for other conditions and were sent home without treatment for the amoebae. Of the adults, one was a neurologic patient whose diarrhea seemed to accompany periods of mental stress. To rule out a possible *histolytica* infection the examination was made which revealed the presence of *Dientamoeba fragilis*, the only contaminating protozoa. This patient became dissatisfied and left before amoebic treatment was begun. The other 3 patients gave histories of definite abdominal distress with accompanying diarrhea. Examination showed *Dientamoeba fragilis* infection in all instances. Treatment with chiniofon in 2 persons and emetine hydrochloride in the third completely cleared up the symptoms, and the stools have remained free from the organisms.

#### DISCUSSION

It appears evident that *Dientamoeba fragilis* is at times the etiologic agent in the type of diarrhea mentioned in the first part of this paper. Almost no work has been done to determine the extent of its pathogenic role. It is not believed to be a tissue invader but stands as an intermediate between the invasive *Endamoeba histolytica* and the nonpathogenic amoebae, such as *Endamoeba coli*.

*Dientamoeba fragilis* is amenable to treatment with any of the drugs used in the control of *E. histolytica*. Emetine hydrochloride has been used with success,<sup>13</sup> but it is undoubtedly a more drastic measure than the disease warrants. Symptoms disappear and organisms become scarce after forty-eight hours of treatment with any of the arsenical or oxyquinoline compounds used for *En-*

*damoeba histolytica*. The full recommended course usually gives complete cure. There may be a recurrence of symptoms due to either an exacerbation or a reinfection, but additional treatment soon brings them under control.

#### CONCLUSIONS

1. *Dientamoeba fragilis* is an amoeba which deserves more attention than it has been awarded.

2. The recognition of this organism is not difficult if its special characteristics are borne in mind.

3. The use of the iron hematoxylin stain facilitates the differentiation of this form from the other amoebae and from vegetable organisms, and thereby helps to confirm the diagnosis made from the saline emulsion.

4. The frequency of occurrence of *Dientamoeba fragilis* at least equals that of *Endamoeba histolytica*.

5. This amoeba undoubtedly produces gastrointestinal symptoms and apparently, at times, an elevation in the eosinophile count.

6. *Dientamoeba fragilis* is amenable to treatment by the same drugs used in the control of *Endamoeba histolytica*.

I am indebted to Dr. R. W. Keeton, head of the Department of Medicine, University of Illinois College of Medicine, for his valuable suggestions and criticisms.

*Note.*—Since the submission of this paper, parasitic examinations have been made on 273 patients. Of this number 13 were found positive for *Dientamoeba fragilis* and 13 were positive for *Endamoeba histolytica*, making a 4.6 per cent infestation for each. These figures are comparable to those given in this paper.

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## FUSION BEATS\*

### A REPORT OF A CLINICAL INSTANCE AND AN EXPERIMENTAL STUDY IN THE DOG

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IN 1911 Lewis<sup>1</sup> published the first curves demonstrating the experimental production of fusion beats ("transitional complexes"), and in 1913 he<sup>2</sup> described a clinical instance of this phenomenon. He regarded fusion beats as the ventricular complexes produced by the ventricles responding partly to the sinoauricular impulse and partly to an ectopic impulse. Variations in the shape of such ventricular complexes were seen in the electrocardiogram, ranging in contour from almost a pure sinus QRS-T to almost a pure ectopic form of QRS-T. Recently, Hill and Cameron<sup>3</sup> recorded similar clinical observations.

Although isolated fusion beats have been noted frequently in electrocardiograms since Lewis' original communication, a series of such beats, representing extrasystoles in various degrees of fusion with the normal sinus impulse, is rare. It has been observed at the Michael Reese Hospital only twice among 10,000 electrocardiograms. For this reason, the analysis of a case demonstrating this phenomenon is reported, and its mechanism analyzed on the basis of an experimental reproduction of this condition in the dog.

#### CASE REPORT

J. C., a white male lawyer, aged 76 years, was first seen on July 15, 1936, complaining of retrosternal pain. He had been in excellent health until six months previously, when progressive weakness and tiredness set in. On June 24, following three strenuous days in conference, he was seized with mild cramping retrosternal pain which persisted for thirty minutes, subsiding on rest. The present attack, similar to the preceding one, occurred one hour after a large lunch, and was accompanied by nausea and by vomiting of undigested food; it lasted one hour and disappeared after resting. The further history was noncontributory.

Physical examination revealed a well-developed, well-nourished, apprehensive male. The chest was moderately increased in its anteroposterior diameter. The lungs were hyperresonant and the breath sounds were somewhat suppressed throughout. A faint apical impulse was visible at the nipple line in the fifth interspace. The cardiac borders were within normal limits. The pulse was 88 per minute. The regular sinus rhythm was interrupted by frequent premature systoles. A faint, blowing systolic murmur was audible at the apex, and a similar, though louder, murmur was heard at the second right and third left interspaces near the sternal border. The liver edge descended 1 cm. below the costal margin in the midclavicular line on inspiration; it was smooth and nontender. The blood pressure was 140 systolic, and 60 diastolic; the temperature, 98° F. (oral); the respirations, 15 per minute. The rest of the examination, urinalysis, and blood count were normal.

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An electrocardiogram (Fig. 1), made one and one-half hours after the cessation of pain, disclosed the following: Rate 91. P-R interval 0.20. QRS upright in limb leads, slurred and small, with  $S_1$  present.  $T_1$  and  $T_2$  inverted.  $T_2$  and  $T_3$  small.  $T_3$  up.  $S-T_1$  depressed.  $S-T_2$  and  $S-T_3$  isoelectric.  $QRS_4$  ( $CF_2$ ) almost entirely down. P, inverted,  $T_4$  diphasic,  $S-T_4$  elevated (but within normal limits). There are frequent ventricular extrasystoles. In Lead II there is a run of four such extrasystoles, three of which are fusion forms of varying degrees. Such fusion beats are also seen earlier in Lead II and in Lead III. Sinus rhythm. Low "voltage." Definitely abnormal curve. Suggests chronic coronary insufficiency.

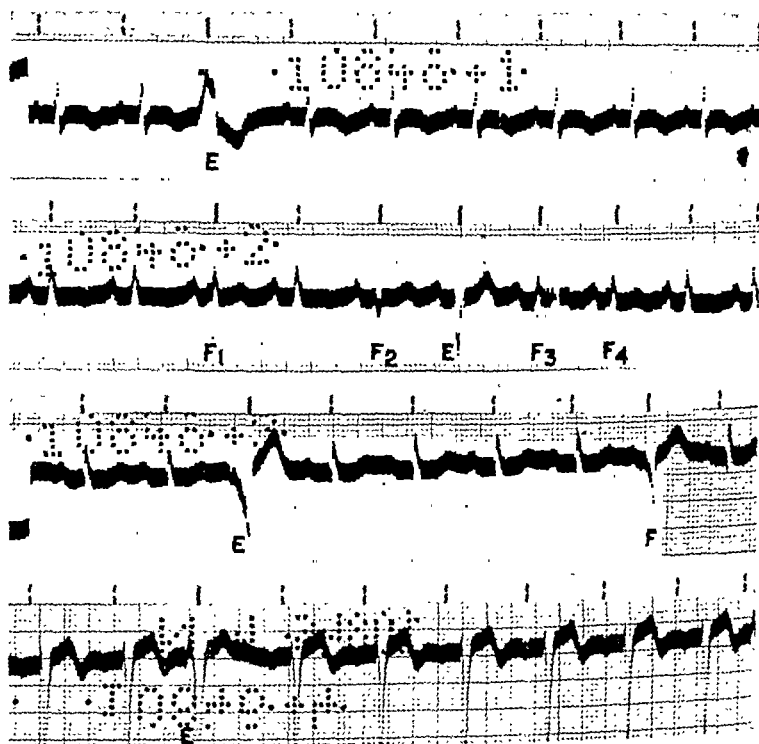


Fig. 1.—Electrocardiogram taken on July 15, 1936. Lead IV is  $CF_2$ . Fusion beats are designated as  $F$ ; ectopic beats as  $E$ . Above each record the average R-R interval of the ectopic rhythm is marked off. Solid lines indicate effective ectopic impulses. Dotted lines represent ineffective ectopic impulses, and demonstrate that the ineffectiveness of the ectopic stimuli is due to "exit" block in Leads I and IV, and to "interference" in Leads II and III. In Lead II fusion beats,  $F_1$ ,  $F_2$ ,  $F_3$ , and  $F_4$  represent varying degrees of fusion between the sinus and ectopic impulses. Description in text.

Clinical diagnosis: (1) Coronary sclerosis. (2) Atheromatous aorta. (3) Chronic pulmonary emphysema.

On the following day, July 16, a presystolic gallop rhythm was audible at the apex. Henceforth, mild brief attacks of retrosternal pain recurred once or twice daily, and premature systoles were noted frequently. On July 22, three severe nocturnal seizures of pain associated with slight dyspnea occurred at two- to three-hour intervals, each lasting fifteen to thirty minutes. During one seizure the pain radiated down both arms to the fingers. The temperature remained normal and the pulse and blood pressure were unchanged. The patient was kept at bed rest for six weeks during which the anginal attacks decreased in frequency and intensity. Since then, mild attacks have appeared after severe exertion or following a large meal, and have been relieved by rest and nitroglycerin (0.0006 Gm.). On Nov. 20, 1936, an electrocardiogram revealed rate averages 82 and P-R interval 0.20 (Fig. 2). Compared with the first record QRS is taller in Leads I and II. Contour of QRS in Lead III has changed.  $T_1$  smaller,  $T_2$  now up,  $T_3$  taller,  $S-T_2$  now depressed.  $S-T_3$  more elevated (still within normal limits).  $T_4$  now upright and has a

longer and smaller negative phase. Frequent ventricular extrasystoles from two foci. The second one in Lead III is interpolated with a prolongation of the succeeding P-R interval to 0.28 second and aberration of QRS-T complex. In Lead IV the last post-extrasystolic beat has a different type of P wave, indicating a shift in the pacemaker. Sinus arrhythmia. Definitely abnormal curve. Shows a progression of the coronary insufficiency.

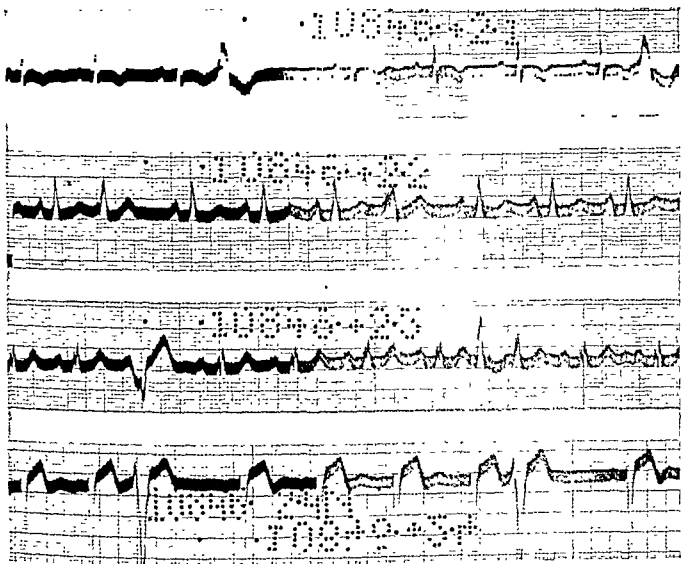


Fig. 2.—Electrocardiogram taken on Nov. 29, 1936. Description in text.

On Oct. 22, 1937, the patient's general condition was subjectively unchanged. The gallop rhythm had disappeared and the heart was regular. A distant blowing diastolic murmur was now audible along the left border of the sternum, and the systolic murmur at the aortic area had become high pitched and musical. The blood pressure was 160 systolic, and 90 diastolic. The new findings were interpreted as indicating slight aortic incompetency on an arteriosclerotic basis, probably with a plaque on one or more of the aortic leaflets. The diastolic murmur has gradually increased in intensity, and premature systoles have reappeared occasionally. Another electrocardiogram was made on Nov. 29, 1938 (Fig. 3). It showed rate averages 78, and P-R interval 0.22 second. Record differs from the one 2 years before in that  $T_1$  is smaller. S-T<sub>1</sub> is now elevated outside of normal range. In Lead V (CF<sub>1</sub>) QRS is inverted and W-shaped, and T is inverted. Sinus arrhythmia. Ventricular extrasystole in Lead II. Fusion beats in Lead I (fifth beat) and in Lead IV (second beat after standardization). First degree auriculoventricular block. Definitely abnormal curve. A progressive chronic coronary insufficiency. At the present writing, his physical condition is unchanged.

#### ANALYSIS OF THE FIRST ELECTROCARDIOGRAM

Measurements were made of the R-R intervals of the sinus beats, and these were compared with the R-R intervals between the ectopic and fusion beats. The R-R intervals of the sinus complexes varied between 0.64 and 0.69 second.

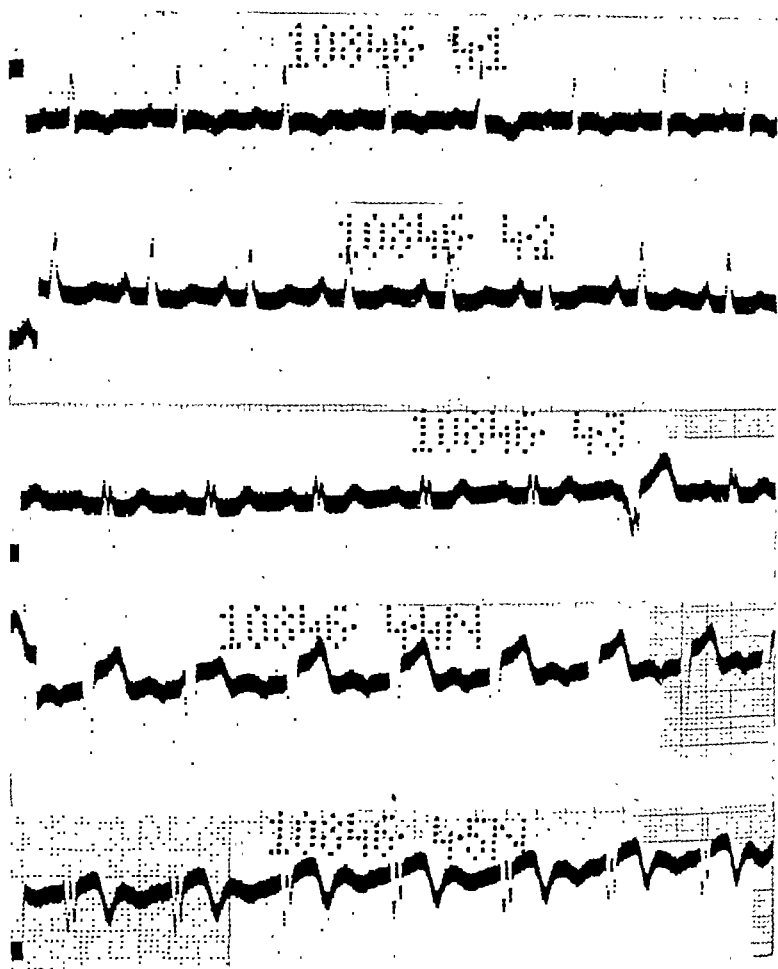


Fig. 3.—Electrocardiogram taken on Nov. 28, 1938. Lead V is  $CF_1$ . Description in text

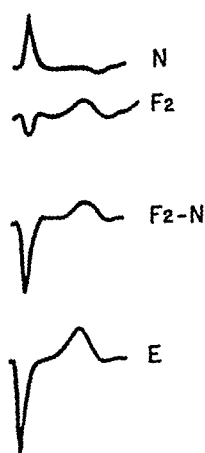


Fig. 4.—Showing the close similarity between an ectopic impulse and the ectopic (rarely systolic) component of the fusion beat.  $N$ , normal ventricular complex.  $F_2$ , fusion beat.  $F_2$ , ectopic impulse. All taken from Lead II of Fig. 1. The curve,  $F_2 - N$ , is the ectopic component of  $F_2$ , constructed by subtracting algebraically the values of  $N$  from  $F_2$  found at corresponding times, using the beginning of the P wave as zero time. Note that the QRS and T waves of the ectopic component,  $F_2 - N$ , are smaller than those of the nonfusion form,  $E$ . Discussed in text.

Similarly, the R-R intervals between the ectopic systoles, shown in Table I, were found to be simple multiples of durations between 0.64 and 0.68 second. From this it appears that the anomalous ventricular complexes occurred at rhythmic intervals, and suggests the presence of two independent rhythms, namely, a sinus and a parasystolic ventricular rhythm. The spacing of the parasystolic rhythm is shown in Fig. 1 by the vertical lines above each lead. The dotted lines show when the ectopic pacemaker fails to affect the ventricle, in some instances because of interference dissociation, in others because of "exit" block.

TABLE I  
R-R INTERVAL BETWEEN PREMATURE SYSTOLES

Lead I	5.92 ( $9 \times 0.658$ )	second
Lead II	1.30 ( $2 \times 0.650$ )	second
	0.64 ( $1 \times 0.640$ )	second
	0.66 ( $1 \times 0.660$ )	second
	0.64 ( $1 \times 0.640$ )	second
Lead III	3.36 ( $5 \times 0.670$ )	second
Lead IV	4.74 ( $7 \times 0.677$ )	second
Average R-R interval		0.664 second

Additional evidence favoring the existence of two independent rhythms giving rise to fusion beats was obtained by retracing on the same scale a sinus beat, an ectopic beat, and a fusion beat, using the beginning of the preceding P wave for zero time in each. The sinus beat was then algebraically subtracted from the fusion beat, and the curve so derived was compared with the ectopic beat. Complexes  $F_1$ ,  $F_2$ ,  $F_3$  and  $F_4$  (Fig. 1) were thus analyzed, and a typical result is shown in Fig. 4, taken from Lead II of Fig. 1. The close similarity between the ectopic (parasystolic) component derived in this manner and the nonfusion forms of premature systoles seen in the electrocardiogram is clearly illustrated by Fig. 4, and offers strong presumptive evidence for the coexistence of the two independent rhythms. The fact that the derived curves (viz.,  $F_2-N$ ) were always smaller than the ectopic complex is not surprising, since the amount of heart muscle stimulated from this focus in the fusion beat is less than when the ectopic beat alone is in control; the amount of ventricle under control of the sinus impulse is also less than usual.

In instances such as this, where the sinus and ectopic rates so closely approximate each other, fusion beats may occur less frequently than anticipated, either because many ectopic impulses occur so late as to fall in the refractory phase of the ventricle (interference), or because the ectopic stimuli are blocked within the pacemaker by a so-called "exit" block. From Fig. 1 it is apparent that in our case, both interference (Leads II and III) and "exit" block (Leads I and IV) were operative, and that, of the effective ectopic impulses, some produced simple premature systoles ( $E$ , Fig. 1), while others combined with the sinus impulse to produce fusion beats ( $F$ , Fig. 1).

#### EXPERIMENTAL PRODUCTION OF FUSION BEATS

Lewis<sup>1</sup>, in his early work on fusion beats, stimulated the left ventricle of a dog by rhythmic shocks induced at a rate slightly more rapid than the sinus rhythm. Ventricular complexes were thus produced, illustrating a gradual

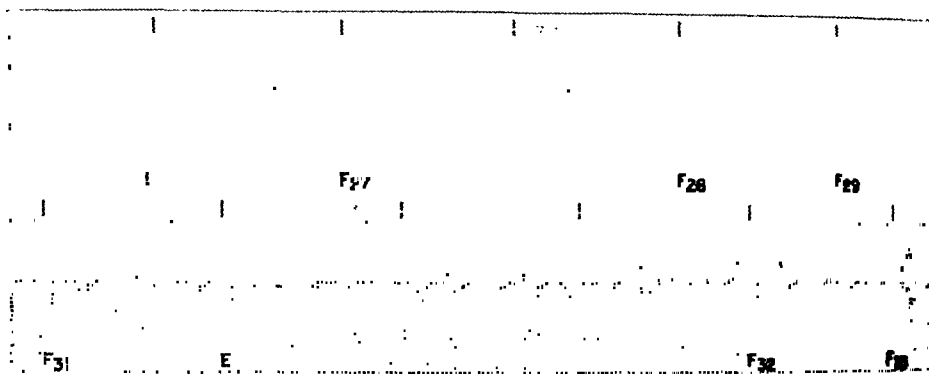


Fig. 5.—Showing some fusion beats produced in the dog (Lead II). *F*, fusion beat. *E*, artificial ectopic beat. The average R-R interval between ectopic stimuli, the rate of the Lewis interrupter, is marked off above each record. Solid lines indicate effective ectopic impulses; dotted lines, ineffective stimuli. (The artificial stimuli were not at all times discharged by the interrupter at absolutely rhythmic intervals, thus explaining the slight variations in the R-R intervals.)

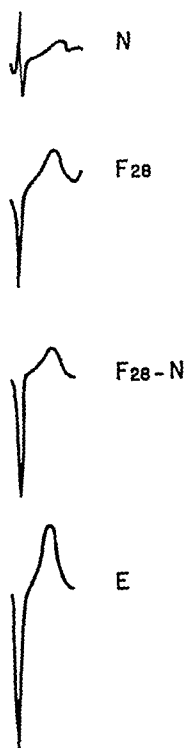


Fig. 6.—Curves illustrating a normal ventricular complex, *N*, in the dog; an experimentally produced fusion beat, *F*<sub>28</sub>; the ectopic component of *F*<sub>28</sub>, constructed as in Fig. 4 by subtracting *N* from *F*<sub>28</sub>; and a representative ectopic impulse, *E*, all drawn from the upper curve of Fig. 5. Note the smaller QRS wave of the ectopic component of *F*<sub>28</sub> as compared with that of *E*. Discussed in text.

transition from the normal sinus complex through a series of intermediate forms of fusion to the pure ectopic form. Later, in a study of bundle branch block, Wilson and Herrmann<sup>4</sup> were able to alter the form of the normal ventricular complexes by electrical excitation of the affected ventricle at a rate either slower or faster than the normal sinus rhythm. Further, by stimulating the affected ventricle at the instant when the auricular impulse reached the

opposite normal ventricle, the composite curve resulting from the summation of electrical effects resembled that of a normal ventricular complex.

Repeating Lewis' method, we studied the mechanism involved in the production of varying degrees of fusion between the sinus impulses and rhythmic ectopic (parasystolic) stimuli. A pair of hooked platinum electrodes were placed on the exposed right ventricle of a dog and with a Lewis interrupter, rhythmic stimuli were induced so as to coincide with every third, fourth, fifth, seventh, or eleventh sinus impulse. The electrocardiogram was recorded using Lead II (see Fig. 5), and the film records were analyzed in the same manner as the clinical record. The ectopic components were again derived mathematically by algebraic subtraction, and a typical example is shown in Fig. 6. The fact that such a known experimental "parasystole" gave rise to fusion beats and to interference dissociation, and that the mathematical derivation of the ectopic component of the fusion beat resembled that in the clinical case, lends further support to the deduction reached in the latter instance. These observations are in close accord with those of Lewis,<sup>1</sup> and Wilson and Herrmann.<sup>4</sup>

Since parasystole with fusion beats is a more benign condition than premature systoles of multiple foci, the differentiation assumes practical importance. Perhaps closer attention to this phenomenon of fusion beats will lead to its more frequent recognition.

#### SUMMARY

A clinical case, manifesting an ectopic ventricular parasystolic rhythm with various degrees of fusion with the normal sinus impulse, is recorded. The mechanism involved in the production of fusion beats is demonstrated experimentally in the dog. Attention is directed to the importance of recognizing fusion beats in the electrocardiogram.

I am indebted to Dr. Louis N. Katz for his advice and guidance in preparing this report, and to Mr. Kenneth Jochim for his assistance in the animal experiment. The interpretations of the electrocardiograms were made by Dr. L. N. Katz.

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## LIPID, CARBOHYDRATE, AND MOISTURE CONTENT OF THE LIVER IN DIABETES MELLITUS\*

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AS A result of histologic study of autopsy material over a period of years Warren<sup>1</sup> has concluded that livers of insulin-treated diabetic patients do not differ radically from those of nondiabetic patients. As a further study of the question it was decided to make chemical analyses on similar tissue, but it was felt advisable to analyze fresh material. Samples of the livers were put into formalin and analyses were carried out after varying lengths of time. The results of these analyses will be presented in another paper.

### EXPERIMENTAL

Autopsies were carried out as soon as permission could be obtained, and the liver samples (fresh and in formalin) were immediately sent to the chemical laboratory. In the case of autopsies performed at night and on Sundays the fresh liver sample was kept in the icebox at 12° C. until the following morning.

The liver was cut into small pieces and mixed thoroughly so as to obtain representative aliquots. About 10 to 12 Gm. each were used for glycogen and free-sugar determinations, between 10 and 15 Gm. for lipids, and about 5 Gm. for moisture.

The method for glycogen as used at present by Dr. G. T. Cori<sup>2</sup> was followed exactly, except that the final glycose obtained after hydrolysis was determined by the Folin-Wu<sup>2</sup> colorimetric method. The Trimble and Carey<sup>3</sup> methods were used for free sugar and for determination of nonfermentable substances. Here also the Folin-Wu method was used for glucose determinations. The samples for moisture determination were dried for twenty-four hours on a steam bath and then in a desiccator over sulfuric acid to constant weight.

The lipid extracts were prepared by the Bloor methods.<sup>4, 5</sup> For determination of total fatty acids and total cholesterol the following method was used: The alcohol-ether extract of lipid was saponified almost to dryness in a 100 c.c. Erlenmeyer flask, covered with a small watch glass. The residue was taken up in a small amount of alcohol and cooled. Volumes of water and petroleum ether, equal to the volume of alcohol, were added, and the petroleum ether layer, containing the nonsaponifiable fraction, was removed in a separatory funnel and washed with a small amount of water, the water being returned to the alcohol-water fraction. The petroleum ether was evaporated, the residue

\*From the Chemical Laboratory, New England Deaconess Hospital, Boston.

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†Personal communication to the author.

TABLE I

RESULTS OF ANALYSIS OF FRESH TISSUE FOR TOTAL FATTY ACIDS, IODINE NUMBER OF THE FATTY ACIDS, PHOSPHOLIPID FATTY ACIDS, TOTAL CHOLESTEROL, AND MOISTURE

CASE NUMBER	SEX	AGE	WEIGHT	WEIGHT OF LIVER	TOTAL FATTY ACIDS	IODINE NUMBER	PHOSPHOLIPID FATTY ACIDS	TOTAL CHOLESTEROL	MOISTURE
				Gm.	Mg. %		Mg. %	Mg. %	%
<i>Diabetic</i>									
37065	M	61	180	2,000	4,031	82.0	1,433	402.0	—
37246	F	64	150	1,630	5,781	80.0	1,766	325.0	—
37307	M	59	175	2,390	10,835	72.0	1,773	431.0	68.0
37564	M	68	115	1,400	3,613	99.0	1,898	332.0	73.9
37612	M	71	150	1,500	3,345	100.0	2,104	420.5	72.5
37666	F	63	125	1,400	3,290	107.0	2,173	312.0	73.0
37876	M	65	140	1,780	2,412	98.0	2,068	266.0	76.2
38230	M	63	157	1,600	3,245	98.0	2,090	279.0	74.5
38267	M	55	140	1,550	3,504	92.0	1,707	164.0	76.4
38424	M	61	140	1,910	2,200	94.0	1,740	220.0	79.2
-----*	F	60	134	1,379	13,779	62.0	3,134	418.0	61.0
38508	F	52	250	1,600	4,203	79.0	2,034	251.0	77.0
-----†	M	68	170	4,472	4,472	77.0	537	46.0	73.6
38782	M	44	180	2,400	2,610	104.0	1,930	238.0	75.7
38994	F	54	260	2,340	2,489	102.0	1,856	227.0	77.7
39056	M	34	150	1,910	2,705	104.0	2,139	252.0	77.5
39416	F	80	220	2,160	15,567	60.0	2,052	412.0	65.0
39438	F	63	185	1,670	3,156	97.5	2,048	325.0	76.2
39439	M	61	155	1,550	2,368	97.0	2,097	241.0	75.9
39566	F	66	116	1,550	10,385	69.7	2,072	315.0	70.8
39781	M	73	145	2,100	3,123	74.0	1,467	209.0	77.6
40055	F	64	1760	1,760	8,928	64.0	2,773	304.0	74.0
40070	F	61	150	2,380	12,183	56.0	1,979	339.0	66.2
<i>Nondiabetic</i>									
36967	M	67	110	1,480	2,432	114.0	1,906	250.0	—
37186	F	59	110	1,000	3,200	113.0	1,624	281.0	—
37235	M	30	125	3,180	4,356	75.2	1,658	266.0	—
37326	M	67	120	2,500	2,207	117.0	1,760	290.0	—
37426	M	48	180	4,000	4,000	121.0	1,851	276.0	—
37463	M	67	140	1,680	3,555	74.0	1,602	313.0	—
37941	F	51	150	1,640	6,297	80.0	2,000	262.0	74.5

\*Liver from biopsy.

†Gastrocnemius muscle.



The phospholipids were very constant and the total cholesterol fairly so, regardless of the total fatty acid values. Both phospholipid and total cholesterol values of the gastrocnemius muscle were very low, the former possibly as a result of inactivity.<sup>7</sup>

In cases where the total fatty acid was approximately normal, e.g., between 2.5 and 4 per cent, there was little correlation between total fatty acid and moisture, but there is an almost mathematical relationship between abnormal fatty acid values and moisture. The lowest total fatty acid percentage found was 2.2 and the highest was about 15.8 per cent, with moisture contents of 79 and 65 per cent, respectively. These results are similar to those of Deuel<sup>12</sup> for rats, and of Kaplan and Chaikoff<sup>13</sup> for dogs.

Table II gives the results of analyses for carbohydrates, in some cases corrected for nonfermentable material. The time that elapsed between the death of the patient and the beginning of analysis is given as "hours post mortem." Also given for comparison are the data on severity of diabetes, the total fatty acid values of the livers, and the last determination of blood sugar. The severity of diabetes is judged by the amount of insulin required to keep the patient in equilibrium. Determinations of carbohydrate were not made on the livers received earlier, and since most of the nondiabetic livers were analyzed at the beginning of the work, no comparisons are possible. It is seen that there was wide variation in both glycogen and free sugar, the nonfermentable material accounting for some, but usually not a great percentage of the apparent value. There was little correlation between carbohydrate values and total fatty acids, severity of disease, time after death, and final blood sugar determinations.

#### DISCUSSION

A diabetic person is one who has a disturbed carbohydrate metabolism; he usually dies from diseases other than diabetes. For this reason, it was felt that the controls should be nondiabetic persons so selected as to represent the different types of disease from which diabetic persons may die rather than normal persons. Thus Case 36967 is a death from carcinoma with an entirely normal liver. Case 37186 is a death from tuberculosis with a liver normal except for slight toxic reaction to the existence of the tuberculous process. Case 37235 is a death from Hodgkin's disease, with some involvement of the liver. Case 37326 is a death from carcinoma of the bladder. Case 37426 is a death from peritonitis, showing the same type of focal necrosis in the liver that so many diabetic patients dying with sepsis show. Case 37463 is a death from a pulmonary embolus and could be regarded as fully normal as a person killed by an automobile instantaneously. Case 37941 is a case of cardiac failure with a normal liver comparable to the cardiac failure so frequently seen in diabetic patients.

A consideration of the results obtained shows that little difference was found between the livers of diabetic and nondiabetic persons that were analyzed.

The large amount of lipid found in a few of the livers apparently consists of neutral fat, which is, as shown by the iodine numbers, of depot nature.

The phospholipid fatty acid values were very constant. Sinclair<sup>14</sup> states: "One of the fundamental concepts concerning the phospholipids has been that

TABLE II

Results of Analyses of the Fresh Tissue for Glycogen and Free Sugar, in Some Cases Corrected for Nonfermentable Material. The "Hours Post Mortem" Given Represent the Time Which Elapsed Between Death and Receipt of the Tissue in the Laboratory. Data on Severity of Diabetes, Final Blood Sugar Determinations, and Total Fatty Acid Values Are Included.

CASE NUMBER	GLYCOGEN		FREE SUGAR		TOTAL CARBOHYDRATE		TIME POST MORTEM		FASTING BLOOD SUGAR*		SEVERITY OF DIABETES	TOTAL FATTY ACIDS
	FOUND	CORRECTED	FOUND	CORRECTED	FOUND	CORRECTED	Hr.	Min.	FOUND	TIME ANTE MORTEM OR POST MORTEM		
	Mg. %	Mg. %	Mg. %	Mg. %	Mg. %	Mg. %			Mg. %			Mg. %
<i>Nondiabetic</i>												
37941	157.6				157.6		1	40				6,297
<i>Diabetic</i>												
38230	315.2		889		1,204.0		2	25	210	3 days ante mortem	Mild	3,245
38267	19.5		110		129.5		10		230	36 hours ante mortem	Moderate	3,504
38424	60.0		112		172.0		4		140	Post mortem	Moderate	2,200
—†	200.2		370		570.0				170	Before operation	Severe	13,779
38508	5.7		140		145.7		9		280	Post mortem	Mild	4,203
—†	921.0		No determinable amount		921.0				250	24 hours before operation	Moderate	4,472
38782	60.0		255		315.0		12		320	Postmortem	Severe	2,610
38994	519.0		745		1,264.0		3		290	24 hours ante mortem	Moderate	2,489
39036	44.5		693		738.0		2		680	Post mortem	Severe	2,705
39416	8.4		376		384.4		2	15	230	3 days ante mortem	Severe	15,567
39438	2,067.0	8.3	685	355	2,752.0	363.3	20	25	750	1 hour post mortem	Moderate	3,156
39439	610.0	1,910.0	848	650	1,458.0	2,561.0	20		140	2 hours post mortem	Mild	2,368
39566	27.8	584.0	240	808	1,120.0	1,392.0	5		230	4 days ante mortem	Moderate	10,385
39781	448.0	262.4	673	414	652.0	676.0	48	30	50	3 hours post mortem	Severe	3,123
40055	192.8	170.0	459	394	2,006.0	516.0	6	30	580	Post mortem	Severe	8,928
40070	1,173.0	1,032.0	833	767		1,819.0	50		360	4 hours ante mortem	Severe	12,183

\*Analyses made in the routine laboratory. In some cases the hours post mortem were not given.

†Blebs of liver.

‡Gastrocnemius muscle.

§Patient had never been treated for diabetes. She was admitted in coma and died nineteen hours later.

the amount of phospholipid in any one tissue is a fixed characteristic of that tissue, much as its histological structure is fixed." According to Abrami,<sup>15</sup> the phospholipid of liver is not appreciably altered by injecting insulin.

The total cholesterol was fairly constant but showed some tendency to vary with the total fatty acids. Phospholipid and free cholesterol values have been shown to maintain a relatively fixed ratio to one another.<sup>14</sup> Ester and free cholesterol determinations were made on the liver extracts of 3 persons with diabetes and of 2 normal persons. Total phospholipid, as well as phospholipid fatty acids, was determined in some cases, and it was found, in agreement with Haven<sup>16</sup> and others, that the fatty acids made up about two-thirds of the molecule. Using this factor and converting the fatty acid values to phospholipid, the average phospholipid:free cholesterol ratios in these 5 cases were 17.2 and 16.8 for diabetic and nondiabetic tissue, respectively. The average phospholipid:total cholesterol ratios for all livers analyzed were  $10.6 \pm 2.2$  and  $9.6 \pm 1.2$ , with ranges of 5.3 to 17.2 and 7.7 to 11.4, respectively. Thus there was greater variation in the diabetic group but fairly close agreement in the ratios between the two groups.

In interpreting the results of carbohydrate analyses several factors must be considered. In a few cases (e.g., Cases 39416 and 39438) there appears to be evidence of fat-glycogen antagonism, but there are many exceptions. The time after death has had no constant effect on carbohydrate values. This is unusual in animal work, but Warren<sup>1</sup> quotes from the work of Popper and Wosazek showing that there was but little change in total carbohydrate in livers of persons with diabetes over a period of two days, regardless of the temperature at which the liver was kept. In the present work there are 4 livers with high carbohydrate values analyzed twenty hours or more post mortem. Liver injury has been shown to lower the amount of glycogen stored.<sup>1</sup> In this study each of 8 liver samples (excluding the biopsy sample) contained more than 500 mg. per cent total carbohydrate. In 6 of these cases the patients showed no clinical or pathologic evidence of liver injury. Sex has been shown by Okey and co-workers<sup>17, 18</sup> and by Deuel,<sup>19-21</sup> to play a part in carbohydrate and fat metabolism in rats. Glycogen content was higher in livers of male, and total fatty acids were higher in livers of female animals. However, of the 8 livers previously mentioned 4 were from male and 4 from female patients; and in one case, a female, the fatty acids were also high. There is little or no correlation between severity of disease or between the last fasting or the post-mortem blood sugar values and liver carbohydrate. The hypothesis is offered that the pathologic conditions which induced death may have affected the enzyme systems.

#### SUMMARY

In an attempt to determine whether differences exist between tissues of diabetic and nondiabetic persons a series of human livers from autopsy were analyzed for lipids, moisture, glycogen, and free sugar.

The results do not show any outstanding differences, except that there was more variation in total fatty acids in the diabetic group. One-third of the livers of persons with diabetes, and 1 out of 7 livers of normal persons, contained more than 5 per cent total fatty acids. Iodine numbers in both groups showed that

the excess fat was of depot nature. The phospholipid fatty acids and total cholesterol values were fairly constant. As liver fatty acids increased above normal the moisture decreased.

The glycogen and free sugar values varied greatly, but showed little or no relationship to severity of the disease, recent blood sugar determinations, total fatty acids, and in some cases, to time after death.

It is a pleasure to acknowledge the helpful interest and advice of Dr. Shields Warren throughout the course of these experiments. I wish to thank Professor W. R. Bloor for his help in interpreting some of the results, and Dr. H. C. Trimble for his interest and suggestions.

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## INFLUENCE OF VARIOUS FAT-CONTAINING DIETS ON THE REACTION OF HISTAMINE ACID PHOSPHATE IN THE SKIN OF WHITE RATS\*

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**S**TUDIES were undertaken to determine whether the cutaneous reactions in white rats to 1:1,000 histamine acid phosphate were influenced by the amount of fat ingested. The work outlined here is based on the urticarial reaction of the skin to histamine applied intradermally, and the results are compared with those obtained in normal control cases.

The histamine wheal was first described by Eppinger<sup>1</sup> in 1913. Dale and Richards<sup>2</sup> made an extensive investigation of the pharmacologic action of histamine in 1918 and concluded that it caused dilation of the capillaries by decreasing the tone of these vessels. Lewis<sup>3</sup> showed that the normal skin gives a constant reaction to histamine characterized by the formation of a central wheal and surrounding flare. He and his associates proved this reaction to be of a threefold character, terming it "the triple response." Starr<sup>4</sup> applied this histamine reaction to a study of the circulation in the feet of patients with diabetic arteriosclerosis and found it impaired or absent in cases with the presence of actual gangrene. The test has been further used by Caldwell and Mayo,<sup>5</sup> Rondelli,<sup>7</sup> Roxburgh,<sup>8</sup> King,<sup>9</sup> and others in testing various reactions of the skin.

Thirty-six 21-day-old white male rats were used in the study. Twelve of these were placed on a high fat diet in group I, 12 on a low fat diet in group II, and 12 on a normal control diet in group III.

Group I, consisting of the rats receiving a high fat diet, were fed Merck's fat-free casein (0.84 per cent fat) 16 per cent, chemically pure sucrose 43 per cent, lard 25 per cent, fat-free dried yeast 12 per cent, and salt (Osborn-Mendel mixture) 4 per cent; vitamins A and D were supplied from nonsaponifiable material extracted from high grade cod-liver oil. Group II received a low fat diet containing Merck's purified fat-free casein (0.84 per cent fat) 16 per cent, chemically pure sucrose 68 per cent, fat-free dried yeast 12 per cent, salt (Osborn-Mendel mixture) 4 per cent, and vitamins A and D in the form of nonsaponifiable material obtained from high grade cod-liver oil. Group III received a normal diet containing Merck's purified fat-free casein (0.84 per cent fat) 16 per cent, chemically pure sucrose 58 per cent, lard 10 per cent, fat-free dried yeast 12 per cent, and salt (Osborn-Mendel mixture) 4 per cent; vitamins A and D were supplied from nonsaponifiable material obtained from high grade cod-liver oil.

The animals were maintained on this dietary regime for sixty-six days, at which time it was felt sufficient changes in the body fat had occurred to make

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observation worth while. We were careful to observe that no obvious fat deficiency changes had become evident.<sup>10</sup>

The skin was tested after the manner of Starr on the sixty-sixth day after the dietary regime was begun; this consisted of the application of one drop of a 1:1,000 aqueous solution of histamine acid phosphate pricked into the skin with seven pricks of a needle. The tests were made on the previously shaved abdominal walls of each of the three groups, and the reactions were read at two and one-half-, five-, and ten-minute intervals.

A normal reaction as seen in group III consisted of a wheal 3 mm. by 3 mm. in diameter, which appeared within two and one-half minutes accompanied by a flare approximately 1 cm. in diameter surrounding the wheal. In group I, or the high fat group, the wheal was 3 mm. by 3 mm. in diameter, with a surrounding erythema 1.5 cm. in diameter; in group II, the low fat group, the wheal was 3 cm. by 3 cm., with an erythematous flare 1.5 cm. in diameter. At times irregular boundaries of the flare were difficult to determine, but this was true in all groups and the flares were essentially the same. The reactions were again tested by injecting intradermally 0.05 c.c. of 1:1,000 histamine acid phosphate. Although the reaction was some larger in each of the three groups, there were no essential differences.

From these observations it appears that the cutaneous reaction in white rats for 1:1,000 histamine acid phosphate with skin prick and intradermal injections is not influenced by the amount of fat ingested in the diet.

We are indebted to Mrs. Susan Gower Smith for supplying the diets used and for many valuable suggestions.

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# THE RELATIONSHIP BETWEEN PLASMA PROTEINS AND ERYTHROCYTE SEDIMENTATION RATES IN CHRONIC ATROPHIC ARTHRITIS\*

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NO UNIFORMITY of opinion exists regarding the factors which are responsible for the increased erythrocyte sedimentation rates observed so frequently in atrophic arthritis. Some investigators conclude that increases in erythrocyte sedimentation result from quantitative changes in the plasma proteins, while others contend there is no causal relationship between the erythrocyte sedimentation rate and the plasma protein concentrations. Patients having atrophic arthritis often show striking clinical improvement following whole blood transfusions with lowering of the sedimentation rate. If changes in sedimentation are quantitatively related to changes in plasma protein concentration, the lowered sedimentation rates observed following transfusion should be reflected in plasma protein estimations.

The investigations here reported were made in the hope of contributing information which might help in the solution of this problem.

## MATERIAL

The investigations were made on patients who were under observation in the Desert Sanatorium, Tucson. The report covers only studies made on patients having a clinical diagnosis of chronic atrophic (chronic infectious) arthritis. Patients having mixed arthritis or hypertrophic arthritis are excluded. The ages of the patients range from 20 years to 71 years, and the duration of the arthritis from 2 years to 40 years. In addition to the patients presenting "active" arthritis, observations made on 8 patients, who had previously had atrophic arthritis but who were clinically arrested at the time of observation, are presented.

## METHODS

Blood was withdrawn from patients by venepuncture, in every instance before breakfast, great care being taken to avoid venous stasis.<sup>1</sup> From the specimens thus obtained the following estimations were made: complete blood count (including erythrocytes, hemoglobin determination, total leucocytes, differential count, and Schilling hemogram); sedimentation rate; plasma albumin, globulin and fibrinogen; albumin-globulin ratio; and total proteins. The Wintrobe

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method<sup>2</sup> was used for hemoglobin determinations. Sedimentation rates were determined by the Westergren method. Three per cent sodium citrate was used as the anticoagulant in the proportion of 0.25 c.c. to 1 c.c. of blood. The observations were begun shortly after the blood was withdrawn, and the tubes were mounted in a rack having a gauge to insure their being vertical. No correction was made for cell volume or temperature. The recorded rate shows the amount of sedimentation that occurred in one hour.

The separation of blood cells from plasma (or sedimentation) is apparently dependent on rouleaux formation. In normal or slowly sedimenting bloods, each rouleau contains few erythrocytes, and the cell aggregates appear evenly distributed. In pathologic bloods showing rapid sedimentation, each rouleau contains many corpuscles, and the rouleaux are clustered together leaving large areas of free plasma between them. The sedimentation rate is directly proportional to the size of the cell aggregates or rouleaux. Since sedimentation depends on the size of the rouleaux and not upon the total volume of cells present, there is no need for cell volume correction. Anemia per se has a negligible effect on the rate of settling of the erythrocytes.<sup>3, 4</sup> Cell volume may affect readings made during the packing stage, but with Westergren tubes of 200 mm. length the sedimentation phase is rarely complete in one hour,<sup>5</sup> at which time readings were made in these studies. Although all observers agree that the temperature of the room or the receptacle influences the sedimentation rate, and great differences in rates are observed when readings are made at temperatures of 10°, 23°, and 37° C.,<sup>6</sup> Wintrobe<sup>7</sup> showed mean variations of only 3 mm. when readings are made at temperatures between 20° and 26° C. Summer temperatures in Tucson frequently reach 37° C., and occasionally 40° C. It has, therefore, been necessary in some of our studies to create artificial cooling. The observations here reported were made at temperatures between 20° and 24° C.

Plasma proteins were estimated by the Wu and Linn<sup>8</sup> colorimetric method. Duplicate determinations were made frequently to insure accuracy of the results.

TABLE I  
NORMAL CONTROLS

CASE	SEX	SEDIMENTATION RATE	TEMPERATURE C°	FIBRINOGEN	ALBUMIN	GLOBULIN	A-G. RATIO	TOTAL PROTEINS
C-7	M	2	24	0.2	5.6	2.7	2.08	8.30
C-10	F	8	22	0.2	4.6	2.4	1.91	7.00
C-10	F	17	22	0.22	5.1	2.26	2.21	7.36
C-11	M			0.29	5.15	2.37	2.17	7.52
C-12	M			0.36	4.71	1.90	2.47	6.61
EH	F			0.37	5.14	3.00	1.71	8.14
FT	M			0.41	4.71	2.05	2.30	6.76

#### RESULTS

Table I shows the results in normal controls. The data include sex, sedimentation rate, plasma fibrinogen, albumin, globulin, albumin-globulin ratio, and total proteins. These figures agree with normals reported by Salvesen.<sup>9</sup>

Table II shows the results in active, moderately severe to severe atrophic arthritis. The data include sex, duration of arthritic symptoms, clinical activity graded 1 to 4, sedimentation rate, plasma fibrinogen, albumin, globulin, albumin-



globulin ratio, and total proteins. To facilitate study of the table, the highest sedimentation rate observed is recorded at the top and progressively diminishing sedimentation rates follow.

TABLE II  
CLINICALLY ACTIVE ATROPHIC ARTHRITIS

NAME	SEX	DURATION OF AR- THRITIS (YR.)	CLIN- ICAL ACTIV- ITY GRADE	SED. RATE MM.	TEMP. C°	FIBRIN- OGEN	ALBU- MIN	GLOB- ULIN	A.-G. RATIO	TOTAL PRO- TEINS
W. E.	F	4	4	125	20	0.55	5.77	2.33	2.4	8.1
W. E.	F	4	4	113	25	0.74	4.58	3.48	1.31	8.06
W. E.	F	4	4	110	24	0.63	5.34	3.04	1.75	8.38
M. D.	F	4	4	110	22	0.68	6.3	0.5	12.6	6.8
M. J.	M	5	4	105	23	0.66	4.62	3.60	1.28	8.22
K.	F	2	3	103	23	0.50	4.15	3.16	1.31	7.31
L. K.	M	7	4	94	22	0.44	6.00	1.43	4.2	7.43
J. E.	M	12	4	89	23	0.80	7.10	0.80	8.8	7.90
C.	M			88	22	0.54	3.60	3.16	1.14	6.76
C. B.	M	7	4	87	22	0.40	5.10	2.30	2.21	7.40
M. J.	M	2	4	84	22	0.63	5.56	2.84	1.95	8.40
M. D.	F	4	4	83	20	0.41	4.28	2.52	1.7	7.80
W. B.	F	25	3	80	23	0.32	4.80	2.37	2.02	7.17
S. J.	F	2	2	75	24	0.66	4.53	3.95	1.15	8.48
A. M.	F	3	4	69	22	0.45	4.62	2.40	1.92	7.02
W. B.	F	25	3	65	25	0.68	5.04	4.90	1.03	9.94
M. J.	M	5	4	65	23	0.50	4.49	3.63	1.22	8.12
M. J.	M	5	4	65	19	0.39	2.37	3.85	1.62	6.22
B. F.	M	12	3	64	21	0.43	4.66	0.31	15.0	4.97
S. J.	F	2	2	62	22	0.43	4.71	5.05	0.93	9.76
M. D.	F	4	4	60	19	0.47	3.85	3.79	1.01	7.64
G. H.	M	40	1	60	22	2.00	4.90	1.50	3.26	6.40
M. M.	M	27	3	60		0.38	5.80	3.0	1.7	8.80
J. H.	M	12	3	60	23	0.50	4.60	1.70	2.7	6.30
S.	F	10	4	57	25	0.41	4.30	3.30	1.33	7.60
A. M.	F	3	4	55	23	0.52	5.35	2.84	1.53	8.19
M. D.	F	4	4	53	23	0.61	6.0	0.55	10.9	6.55
W. R.	M	8	3	50	23	0.49	4.92	2.68	1.83	7.60
W. R.	M	8	3	50	23	0.41	4.92	2.37	2.08	7.29
J. H.	M	12	3	50	21	0.42	3.80	3.26	1.16	7.06
S. J.	F	2	2	50	22	0.63	5.56	2.84	1.95	8.40
G. M.	M		1	49	22	0.40	5.80	3.00	1.93	8.80
J. H.	M	12	3	47	22	0.44	4.19	2.21	1.89	6.40
B. C.	M	6	2	42	24	0.36	4.30	2.84	1.5	7.14
R. C.	F	30	1	40	20	0.40	5.20	2.30	2.26	7.50
C.	F	$\frac{1}{2}$	1	40	22	0.23	5.20	3.0	1.70	8.20
L. K.	M	7	4	39	21	0.40	4.45	3.22	1.38	7.67
J. E.	M	12	3	35	24	0.51	3.80	1.60	2.3	5.40
I. L.	F	$\frac{1}{2}$	1	33	23	0.33	4.30	3.16	1.34	7.46
G. M.	M		1	31	22	0.47	4.91	3.31	1.5	8.22
I. L.	F	$\frac{1}{2}$	1	30	23	0.34	3.42	2.43	1.4	5.85
J. E.	M	12	2	25	21	0.34	3.85	2.84	1.37	6.69
I. L.	F	$\frac{1}{2}$	1	20	20	0.38	2.48	4.04	0.61	6.52

*Fibrinogen:* While fibrinogen increase is observed in the majority of these patients and high sedimentation rates are frequently accompanied by high plasma fibrinogen content, there are numerous exceptions to the rule, and some of the highest sedimentation rates are observed in bloods which show relatively little increase in fibrinogen. In some rapidly settling bloods no increase in fibrinogen is observed, whereas, considerable fibrinogen increase is observed in bloods showing only moderately increased sedimentation.

TABLE III  
 REPEATED STUDIES  
 Patient D-15, Female

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	A-G. RATIO	TOTAL PROTEINS	R. B. C. MILLIONS	Hb. GM.	W. B. C.	NEUTROPHILES (SCHILLING)	SED. RATE MM.	TEMP. C.°
1937											
10/22						4.448	12.4	10,800	0/0/0/62	110	26
11/17						4.240	13	11,700	0/0/0/53	120	22
1938											
1/3						4.240	11.5	9,300	0/0/1/61	96	21
2/14						4.590	13.4	12,000	0/0/2/63	77	23
3/25						4.760	13.6	6,800	0/0/0/61	75	23
4/26						4.320	12.2	10,600	0/0/2/66	90	25
5/18						4.320	12.2	9,100	0/0/1/56	110	23
5/19*	0.68	6.3	0.5	1.26	6.8						
5/36*											
6/2*											
6/8						4.700	14.2	10,000	0/1/2/59	65	22
6/11*											
6/24*											
7/6											
7/9						5.040	15	10,900	0/0/1/60	53	20
8/6						5.040	15	10,900	0/0/1/60	53	20
9/3		6.0	0.55	10.9	6.55	4.510	13.4	9,600	0/0/1/70	91	24
10/4						4.520		9,400	0/0/0/63	86	24
11/4	0.41	4.28	2.52	1.7	7.80	4.480	12.8	9,100	0/0/2/73	83	20
	0.47	3.85	3.79	1.01	7.64	4.590	13	9,800	0/0/10/59	60	19

\*Blood transfusions given.

TABLE IV  
 REPEATED STUDIES  
 Patient E-16, Male

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	A.-G. RATIO	TOTAL PROTEINS	R. B. C. MILLIONS	HB. GM.	W. B. C.	NEUTROPHILES (SCHILLING)	SED. RATE MM.	TEMP. C.°
1038											
7/13	0.8	7.1	0.8	8.9	7.90	4.200	10.6	7,850	0/0/0/76	89	23
7/22*											
7/28†											
8/5†											
8/6*											
8/12†											
8/17*											
8/20†	0.51	3.8	1.6	2.3	5.40	5.100	13	7,700	0/0/0/69	35	24
8/20	0.34	3.85	2.84	1.37	6.69					25	21
9/20											

\* Aspiration of right knee joint effusion.

† Blood transfusions of 300 c.c. each.

Note. Daily temperature to 100° F. on admission.

Normal temperature from September 3 to September 20.

TABLE V  
REPEATED STUDIES  
Patient K-27, Male

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	A-G. RATIO	TOTAL PROTEINS	R. B. C. MILLIONS	Hb. GM.	W. B. C.	NEUTROPHILES (SCHILLING)	SED. RATE MM.	TEMP. C.°
1938											
3/9						4.910	13.2	10,000	0/0/1/68	85	23.5
4/5						5.430	14.3	10,800	0/0/0/55	79	25
6/6						4.980	14.2	7,400	0/0/0/68	53	22
6/21						5.060	14.4	7,800	0/0/0/67	94	22
6/23*											
6/24		6.0	1.43	4.2	7.43	5.060	14.4	7,800	0/0/0/67	94	22
6/28*											
7/21						5.000	14	9,100	0/0/0/67	45	25
7/28						5.230	15	8,550			
7/29											
7/30*										35	25
8/6*											
8/13											
9/20						5.290	15.2	8,000	0/0/0/72	50	25
10/3		4.45	3.22	1.38	7.67	5.080	14.4	10,000	0/0/0/77	39	21
10/12*	0.40									44	21

\*Blood transfusions of 300 c.c. each.

TABLE VI  
REPEATED STUDIES  
Patient J-55, Female

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	A-G. RATIO	TOTAL PROTEINS	R. B. C. MILLIONS	Hb. GM.	W. B. C.	NEUTROPHILES (SCHILLING)	SED. RATE MM.	TEMP. C.°
1938											
9/16											
9/20*	0.66	4.53	3.95	1.15	8.48	3.830	11	7,400	0/0/0/74	75	24
9/26*											
9/30	0.43	4.71	5.05	0.93	9.76	4.090	13.8	8,400	0/0/1/67	62	22
10/8*											
10/14	0.63	5.56	2.84	1.05	8.40					50	22

\*Blood transfusions of 300 c.c. each.

*Albumin:* In only 6 patients are the albumin concentrations below normal levels. Four of these 6 patients showed normal albumin values at other examinations. The group as a whole shows no tendency to hypoalbuminemia, and no relationship is evident between plasma albumin concentration and sedimentation rate.

*Globulin:* Of the 43 globulin estimations recorded, 24 are above average normals. The globulin increase is always proportional to the increase in sedimentation rate.

*Albumin-Globulin Ratio:* Due to the apparent increase in globulin, a reduced albumin-globulin ratio is the most constant finding in these studies, but no relationship to the sedimentation rates is demonstrated. Some of the lowest ratios observed accompany relatively low sedimentation rates. Albumin-globulin ratios will be discussed later.

*Total Proteins:* Total protein concentration is below normal in only two patients. There is no evident relationship between total protein concentration and sedimentation rate.

Tables III to VIII, inclusive, show a chronologic record of blood counts, sedimentation rates, and repeated protein estimations in individual patients who had received blood transfusions. All these patients had clinically active grade 4 atrophic arthritis with multiple joint involvement.

Patient D-15 (Table III) shows great reduction in sedimentation rate following repeated small transfusions after long-continued high rates and failure to improve clinically on other therapeutic measures. No change in plasma protein percentages or in their proportion to each other accompanied the fall in sedimentation rate. Although the sedimentation rate increased again, the increase was accompanied by a reduction in fibrinogen and normal plasma protein relationships (October 4); a definitely lower sedimentation rate (November 4) was accompanied by an increase in both fibrinogen and globulin.

Patient E-16 (Table IV) definitely shows improvement in plasma protein relations accompanying lowered sedimentation rates. Although plasma proteins were normal at the last examination, the patient at that time showed definite evidence of clinical activity.

Patient K-27 (Table V) was observed for three months before transfusion was given. During that period he failed to make any clinical improvement. Following two transfusions, on June 23 and on June 28, clinical improvement was definite and sedimentation rate was reduced. Low fibrinogen and globulin percentages accompanied a high sedimentation rate. A greatly reduced sedimentation rate (September 20) accompanied increased globulin and diminished albumin with practically no change in fibrinogen percentage. The same total protein concentration was observed with sedimentation rates showing great variation.

Patient J-55 (Table VI) shows only a slight reduction in sedimentation rate accompanied by a relatively great reduction in plasma fibrinogen following two blood transfusions. Definite lowering of the albumin-globulin ratio was observed with the highest sedimentation rate. A lower sedimentation rate (September 30) was accompanied by reversal of the albumin-globulin ratio. The sedimentation rate observed on October 14 is still high, though the albumin-globulin ratio has returned to normal.

TABLE VII  
REPEATED STUDIES  
Patient J-56, Male

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	A.-G. RATIO	TOTAL PROTEINS	R. B. C. MILLIONS	Hb. GM.	W. B. C.	NEUTROPHILES (SCHILLING)	SED. RATE MM.	TEMP. C.°
1938											
9/16	0.66	4.62	3.60	1.28	8.22	4.580	13	4,300	0/0/1/65	105	
9/22*											
9/27*											
9/30	0.50	4.49	3.63	1.23	8.12	4.680	13.4	5,650	0/0/0/51	65	23
10/4*											
10/10*											
10/14	0.63	5.56	2.84	1.95	8.40	4.860	16	5,700	0/0/1/45	84	22
11/10	0.39	3.85	2.37	1.62	6.22	5.240	14	7,900	0/0/20/39	65	22
12/13	0.41	4.71	1.58	2.98	6.29	5.950	17.6	4,600	0/0/19/48	69	23
1/24	0.45	4.92	3.16	1.55	8.08	5.430	14	6,100	0/0/8/39	58	20

\*Blood transfusions.

TABLE VIII  
REPEATED STUDIES  
Patient WE

DATE	FIBRINOGEN	ALBUMIN	GLOBULIN	A.-G. RATIO	TOTAL PROTEINS	R. B. C. MILLIONS	Hb. GM.	W. B. C.	NEUTROPHILES (SCHILLING)	SED. RATE MM.	TEMP. C.°
1939											
1/17	0.55	5.77	2.33	2.4	8.1	4.140	12.8	5,850	0/1/7/46	117	20
3/17	0.63	5.34	3.04	1.75	8.38	5.000	12.0	5,600	0/3/14/49	110	24
4/18	0.74	4.58	3.48	1.31	8.06					113	25

Blood transfusions: Jan. 20, 1939; Jan. 26, 1939; Feb. 3, 1939; Feb. 9, 1939; March 20, 1939; March 25, 1939.  
Tonsillectomy was performed on Feb. 23, 1939.

A relatively high plasma fibrinogen and globulin with a low albumin-globulin ratio accompanied a rapid sedimentation rate in patient J-56 (Table VII). Two weeks later, after two blood transfusions, a reduction in fibrinogen was observed with a considerable lowering of the sedimentation rate. The albumin and globulin fractions and the total proteins remained unchanged. A comparison of plasma protein determinations made on September 30 and November 10, demonstrates the great differences which may occur in total protein, fibrinogen, and the albumin-globulin factors with identical sedimentation rates. Although the sedimentation rates observed on September 30 and November 10 are identical, the plasma fibrinogen, the albumin and globulin factors, and the total proteins are all lower on the latter date.

Table VIII shows repeated estimations of plasma proteins in a patient whose sedimentation rate remained practically unchanged. The record shows only the sedimentation rates done at the times the plasma proteins were estimated. Additional observations of the sedimentation rate made at frequent intervals between the dates shown in the record varied only a few millimeters from those shown here. Four transfusions at intervals of approximately one week failed to reduce the sedimentation rate materially, though the patient's temperature was brought to normal levels and she was clinically improved. Badly infected tonsils, showing a nearly pure culture of an alpha streptococcus, were removed on February 23. With sedimentation rates at the same level, fibrinogen increase was observed following the transfusions. The globulin increased slightly, though the total proteins remained constant. Transfusions were done again on March 20 and on March 25. When the sedimentation rate and plasma proteins were again studied on April 18, further increase in fibrinogen appeared, the globulin showed increase, and there was a reduction in the albumin concentration. A progressive fall in the albumin-globulin ratio was demonstrated while erythrocyte sedimentation remained constant.

TABLE IX  
CLINICALLY ARRESTED ATROPHIC ARTHRITIS

PATIENT	AGE	SEX	DURATION ARTHRITIS YR.	SED. RATE MM.	TEMP. C.°	FIBRINOGEN	ALBUMIN	GLOBULIN	A.-G. RATIO	TOTAL PROTEINS
S-46	24	F	4	20	22	0.6	4.7	2.2	2.13	6.9
P-39	27	F	8	17	27	0.4	5.8	2.9	2.0	8.7
K-26	23	M	4	15	21	0.3	6.0	4.0	1.5	10.0
P-40	24	M	5	14		0.4	6.0	0.42	14.28	6.42
R-44	36	M	4	14	23	0.45	5.2	0.5	10.40	5.70
T	51	M		13	22	0.49	5.13	2.05	2.5	7.18
G-20	48	M	26	8	25	0.45	5.5	1.9	2.36	7.40
-52	32	F		17	22	0.41	4.8	2.6	1.84	7.40

Table IX shows that even years after quiescence of the arthritic process and relatively low sedimentation rates, there often remains some increase in the plasma fibrinogen content and a disturbance in plasma protein relationships.

#### DISCUSSION

It is evident from many studies made that some change in the plasma is responsible for acceleration of the erythrocyte sedimentation rate. When the erythrocytes of a rapidly settling blood are separated from the plasma in which

they are contained and are resuspended in the plasma of a slowly settling blood, they settle slowly.<sup>8</sup> Erythrocytes suspended in a pure albumin solution settle very slowly; when they are suspended in a pure globulin solution, they settle rapidly; in a solution of fibrinogen, they settle very rapidly.<sup>10</sup> When albumin is added to a globulin solution, the sedimentation of erythrocytes suspended in it is not altered, whereas the addition of fibrinogen definitely accelerates sedimentation. When the concentration of globulin is increased, the sedimentation rate is increased, though the sedimentation rate increases more rapidly than the increase in globulin concentration.<sup>11</sup> Some investigators<sup>10, 12-14</sup> consider fibrinogen the most important factor influencing the sedimentation rate, and Ernstene<sup>15</sup> observed definite parallelism between the plasma fibrinogen concentration and the sedimentation rate in rheumatic fever. Others<sup>16-19</sup> find no definite parallelism between fibrinogen percentage and sedimentation rates. In atrophic arthritis divergent opinions have been expressed. Davis<sup>20</sup> reports increase in fibrinogen and globulin proportional to the increase in sedimentation rate, but Aldred-Brown and Munro<sup>19</sup> find no parallelism between sedimentation rate and either plasma globulin content, albumin percentage, albumin-globulin ratio, or total proteins.

There is considerable doubt whether serum albumin and globulin can be regarded as chemical entities<sup>21, 22</sup> or whether separation of the plasma protein into distinct fractions is possible.<sup>23, 24</sup> That portion of the protein which is precipitated by 50 per cent saturation with ammonium chloride is usually considered to represent the globulin fraction. Roche, Dorier, and Samuel<sup>25</sup> find that higher saturation may be necessary to precipitate the globulin from normal sera, and that certain pathologic plasmas have characteristics which make separation of the albumin and globulin impossible.<sup>26</sup> Modification in the molecular constitution of blood protein has been demonstrated in the carcinoma,<sup>27</sup> which also is characterized by a great increase in erythrocyte sedimentation, and in other pathologic states. Tiffeneau and Gysin<sup>28</sup> find that sedimentation rate depends on the size of plasma protein molecules which Svedberg<sup>22</sup> has shown are capable of change in their molecular size. When so much uncertainty exists regarding the separation of plasma protein into distinct fractions, albumin-globulin ratios can have but little, if any, significance. Change in the composition or size of the protein molecules in the plasma of persons with arthritis may account for the divergent results obtained by different workers attempting to explain changes in erythrocyte sedimentation rates on the basis of quantitative change in plasma protein fractions separated by chemical methods.

Cholesterol and the phosphatides may play some part in modifying the effects of the plasma proteins on erythrocyte sedimentation. We made attempts to relate cholesterol content to the observed discrepancies between plasma proteins and sedimentation rates without success.

#### SUMMARY AND CONCLUSION

1. Parallel studies of erythrocyte sedimentation and plasma proteins in patients having atrophic arthritis fail to demonstrate any constant relationship between the sedimentation rate and the percentage concentration of the plasma proteins.



2. Repeated small whole blood transfusions are frequently followed by reduction in sedimentation rate without improvement in plasma protein relationships.

3. The possible reasons for failure to establish quantitative relationship between plasma protein concentration and sedimentation rate are discussed.

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# THE SIGNIFICANCE OF URINARY CHLORIDES IN CASES OF PNEUMONIA TREATED WITH SULFAPYRIDINE\*

WITH SOME REMARKS ON THE SECONDARY TEMPERATURE RISE

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IN 1850 Redtenbacher<sup>1</sup> reported the diminution of urinary chlorides in the course of pneumonia. Since that time considerable literature has accumulated dealing with the pathogenesis and significance of this retention of chloride. The urinary chlorides reach exaggerated low levels just prior to the crisis, with the lowest level occurring at the time of the crisis. One to three days later, however, it rises abruptly<sup>2</sup> to supranormal values, to return subsequently to normal.

As it had been reported that sulfapyridine does not produce all the changes encountered at the spontaneous crisis,<sup>3</sup> it was decided to investigate the chloride metabolism in persons with pneumonia who received sulfapyridine, with the view of correlating the chloride output with either clinical improvement, or a possible clinically undetectable "masked crisis." For this purpose, the chloride content of the twenty-four-hour urinary specimens was determined daily for the entire period of the hospital stay in 17 persons with pneumococcus pneumonia. Of these, 11 were definable as definite cases of lobar consolidation by x-ray studies. The remaining 6 cases were probably also lobar pneumonia, even though the clinical evidence was not sufficiently conclusive to classify them as such. It was found that the chlorides began to rise on the sixth to the fourteenth day of the illness, and from the third to the seventh day after the initiation of sulfapyridine therapy (Table I). In almost every case the summit of the chloride output was reached the day following the initial chloride rise, or, in rare cases, two days later.

Because this chloride rise started after a fairly constant interval of two to six days, with the majority four to five days following the temperature drop, one must consider a possible "crisis" relationship between the chloride output and the defervescence, even though this interval was longer than that usually found in untreated persons with pneumonia. This is further suggested by the findings of oliguria and hypochloruria in rabbits<sup>4</sup> after sulfapyridine, a fact which might account for the additional delay in chloride response after defervescence. It is to be noted in Table I that in those instances in which sulfapyridine was instituted early in the disease, the rise in the urinary chloride occurred at an earlier period in the course of the disease than in those in which it was used at a later period. If the time of chloride excretion was not altered by sulfapyridine, one would expect an inverse relationship between the time of chloride excretion and the duration of the disease when therapy was instituted:

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the interval between therapy and chloride excretion would be greater in the persons treated earlier and shorter in the persons treated later in the course of the disease.

Because of these considerations, it appears that the mechanism responsible for chloride excretion is similar in both the spontaneously and the chemotherapeutically induced crisis, despite persistence of the physical signs and x-ray findings in the latter.

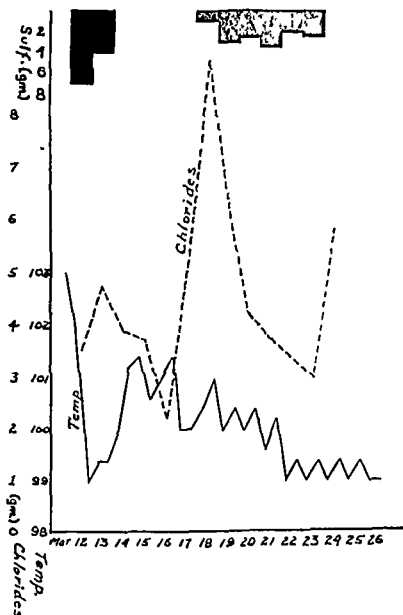


Chart 1.

It is noteworthy to single out the chloride response in persons with secondary complicating infections, in which the primary pulmonary pathology has been arrested, as well as in persons with relapse.

*Case I With Relapse (Chart 1).*—In a case of type IX pneumococcus pneumonia with relapse, the urinary chlorides rose on the tenth day of illness, but dropped again on the twelfth day. This drop continued till the seventeenth day, when it started rising, returning to a normal level on the nineteenth day of illness. During the period of the secondary drop in the chlorides, the patient's complaints recurred, bloody sputum containing the type IX pneumococcus again appeared, and the fever returned. With the second urinary chloride rise, however, the clinical condition improved and the temperature fell and remained normal.

*Case II With Slow Improvement (Chart 2).*—In a case of protracted pneumonia in which the sputum contained a type XI and type XVI pneumococcus, there was a question of independent acute sinusitis. Because of the low chloride, no operative interference was resorted to. The patient began to improve, insofar as pulmonary complaints were concerned, just prior to the rise in chlorides on the twenty-second day of illness, despite the fact that the sinus condition remained stationary.

*Case III With Extrapulmonary Complication (Chart 3).*—In another case of a 2-year-old child the urinary output varied from 0.2 Gm. to 1 Gm. per day. A secondary rise in fever occurred on the sixteenth day of illness, despite the fact that the chlorides had risen to 3 Gm. per day on the fourteenth day of illness, and remained elevated above 2 Gm. thereafter. Since we have found in our cases that the urinary chloride excretion in children under 5 years usually did not rise much above 2 to 3 Gm., this was considered a normal urinary chloride response and, accordingly, another cause for the recrudescence of fever was sought for, and an acute purulent mastoiditis was found. The patient recovered promptly after mastoidectomy.

TABLE I

CASE	AGE-YEARS	TYPE PNEUMOCOCCUS	SULFAPYRIDINE THERAPY			RISE IN URINARY CHLORIDES			SECONDARY RISE OF T.°				REMARKS
			STARTED	STOPPED	DROP OF T.°	DAY OF ILLNESS	DAYS AFTER SULFAPYRIDINE STARTED	DAYS AFTER T.° DROP	DAY OF ILLNESS	DAYS AFTER SULFAPYRIDINE STARTED	DAYS AFTER T.° DROP	DAYS BEFORE CHLORIDE RISE	
1	36	III	2p*	7	3a*	7	5	4	5	3	2	2	Relapse
2	76	IV	2p	4	3a	8	6	5	7	5	4	1	
3	4½	XIV, XV	2p	6	3p	8	6	5	8	6	5	0	
4	9 mo.	XIV	3p	5	4a	8	5	4	7	4	3	1	
5	19	?	3p	6	4p	9	6	5	6	3	2	3	
6	14	III, XIII	4p	6	5a	8	4	3	7	3	2	1	
7	1½	XIV	4p	6	5a	8	4	3	7	3	2	3	
8	58	III	4p	5	5p	10	6	5	7	3	2	3	
9	48	IX	5p	6	6p	10	5	4					
10	71	III	7p	9	8p	14	7	6	14	7	6	0	
11	57	III	8p	10	9a	13	5	4	13	5	4	0	Otitis Mastoid
12	6	?	2p	3	3a	7	5	4	4	2	1	3	
13	10 mo.	XIV	3p	4	3p	7	4	4	6	3	3	1	
14	6½	IV	4a	5	4p	6	3	2		None			
15	6	III	5a	7	5a	11	6	6					
16	9 mo.	VI	10p	12	10p	13	3	3	11	1	1	2	
17	2	?	10a	17	11p	14	3	3					

\*a = A.M.; p = P.M.

#### SECONDARY TEMPERATURE RISE

Thirteen of our patients had a secondary temperature rise varying from 99.7° F. to 103.0° F. after initial defervescence. Five of these occurred on the seventh day of illness, 3 on the ninth, 2 on the eighth, and 1 each on the eleventh, thirteenth, and fifteenth (Table I). It was found that sulfapyridine did not influence this secondary rise. This experience is similar to that of Flippin and

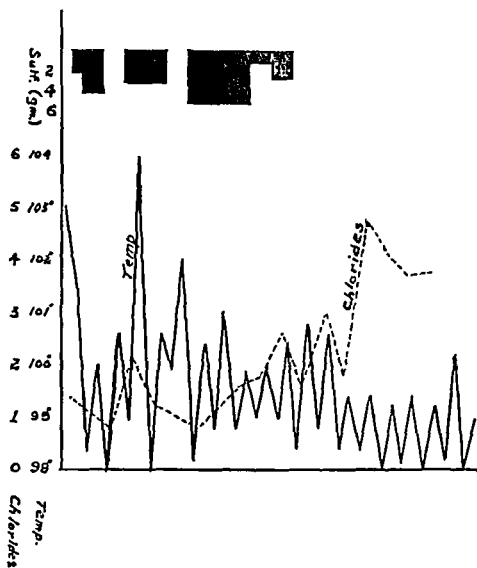


Chart 2.

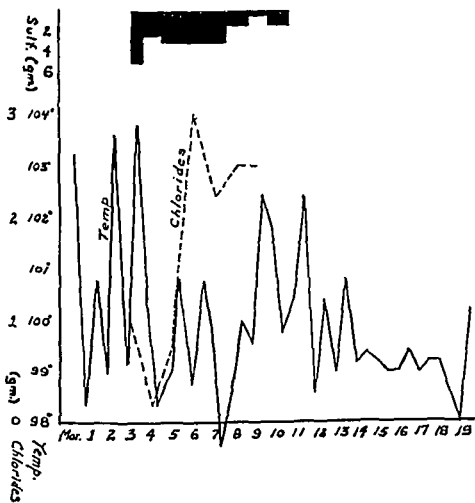


Chart 3.

his collaborators.<sup>5</sup> In 4 of these patients the secondary rise of temperature occurred simultaneously with the rise in urinary chloride, and in 3 patients, one, two, and three days prior to this rise. From the sequential position of these phenomena it might be assumed that there is an apparent relationship between the secondary temperature rise and the urinary chloride increase, corresponding with the "crisis-urinary chloride events," occurring in untreated patients with pneumonia; and that the secondary temperature rise could very well correspond with the precritical hyperpyrexia occurring just prior to the crisis. However, we have found<sup>4</sup> that administration of sulfapyridine to normal rabbits may be followed occasionally by a secondary fever three to four days after the drug has been discontinued: a finding which must also be entertained as a possible explanation which might account for the secondary temperature rise in the human cases.

#### SUMMARY AND CONCLUSIONS

1. The urinary chloride rise in 17 persons with pneumonia treated with sulfapyridine occurred two to six days after defervescence, with the great majority occurring on the fourth or fifth day.

2. The rise is apparently related to a mechanism similar to that occurring after the spontaneous crisis in pneumonia.

3. The rise is uninfluenced by extrapulmonary complications.

4. In 13 of these patients there was a secondary temperature rise after defervescence. This occurred between the day of the increased urinary chloride output and three days before this rise.

5. This secondary rise in temperature is not influenced by sulfapyridine and may be either a "crisis effect," or a delayed reaction to sulfapyridine.

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# CHICKENPOX FOLLOWING CONTACT WITH HERPES ZOSTER\*

## REPORT OF TWO MINOR EPIDEMICS

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A CLINICAL syndrome resembling chickenpox occurred in several patients under our observation at the Children's Division of the Cook County Hospital during the past year.

The original source of the virus in one instance apparently was a bedridden child, M. B., in the tuberculous ward. This child was transferred from the oral surgery ward on March 4, 1938, with a diagnosis of tuberculous osteomyelitis of the mandible. His only contacts on the tuberculous ward were with nurses, attendants, medical personnel, and other patients in his own six-bed room. No visitors were allowed. None of his contacts were known to have had chickenpox or herpes zoster.

On May 27, 1938, eleven weeks after his admission to the ward, he developed two patches of vesicles at the same thoracic-segment level of the chest. One patch was just below the left nipple and the other patch was immediately to the left of the fourth thoracic vertebra. The patches were 3.5 cm. in diameter and consisted of from eight to ten pinhead to small pea-sized vesicles containing a clear fluid. Between these patches, following the course of the intercostal nerve, were two fresh single vesicles on a slightly elevated erythematous base. The posterior patch had been scratched and denuded. There were no constitutional symptoms. A diagnosis of herpes zoster was made; it was confirmed by the division of dermatology and the division of contagious diseases.

On June 9, 1938, thirteen days after the eruption of herpes in M. B., the second child, L. J., who had been in this same room for one year prior to the admission of the child with herpes, developed vesicles on the face and back. This child had Pott's disease of the lower thoracic vertebrae and had been on a Bradford frame since June, 1937. With the exception of being taken to the x-ray department on the day before he developed his eruption, he had had no exposure other than to the personnel in attendance on his ward. Shortly after the first vesicles were noted on the back and face, papulovesicular lesions appeared on the chest and extremities. On advice of a consulting physician from the contagious division, he was transferred with the diagnosis of chickenpox. By June 21, twelve days later, the last crust had disappeared, leaving only a few scars, and the child was transferred back to the tuberculous ward.

A secondary case of chickenpox occurred in a patient, R. V., who had been admitted to the adjacent room on June 4, 1938, five days prior to the onset of chickenpox in L. J. This child had not had chickenpox previously, nor had she had any recent contact with contagious diseases. As in the other rooms on this ward, no visitors were allowed at any time. On June 25, 1938, twenty-one days after her entrance in the hospital and sixteen days after L. J. had developed his lesions, R. V. developed a universally disseminated eruption that passed through the papular, vesicular, and later the crusting stages. She was transferred to the contagious division as a case of chickenpox. There were no further cases.

In the second episode the source of the virus could be traced to a nurse who had herpes zoster. The nurse, who had had chickenpox in childhood, developed an eruption

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at the upper and lower edge of the left breast and over the left scapula on Oct. 11, 1933. The lesions were pinhead-sized vesicles on an elevated erythematous base, and were accompanied by moderate deep pain on the day of onset. Her temperature varied between 98° F. and 99° F. A week after the onset she was referred to a dermatologic consultant, who made a diagnosis of herpes zoster.

Two children exposed to this nurse developed chickenpox-like lesions within eighteen and fourteen days, respectively.

The first child, B. M., was admitted to the hospital Oct. 12, 1933, with a diagnosis of lead encephalitis. She had not had chickenpox, and her parents stated that she had not had any exposure to contagious diseases. She was a bedridden patient in a six-bed room, and was not allowed to have visitors. The nurse who had developed the herpetic eruption on October 11, was on duty in her room. On October 30, eighteen days later, B. M. developed a generalized papulovesicular eruption. It was diagnosed as chickenpox and the diagnosis was confirmed by a consultant from the contagious division, to which she was immediately transferred. None of the children exposed developed chickenpox.

E. D., the second child who developed chickenpox after exposure to this same nurse, was on the same floor as patient B. M., but at the opposite end of the hall. He had been in the hospital for one month with severe bacillary dysentery, and was isolated in a room with other dysentery patients. On October 18 the nurse with herpes zoster was transferred for duty to the dysentery ward. On November 1, fourteen days after the nurse had been transferred to his room, E. D., who was now convalescent and awaiting release stool cultures, developed a temperature of 100° F. and soon after was noted to have a varicelliform eruption on the body. A consultant from the contagious division confirmed the diagnosis of chickenpox, and the child was transferred to that division. It is interesting to note that this child also developed scarlet fever two days after his transfer to the contagious hospital.

In each of these episodes, after chickenpox was diagnosed and the patients were transferred to the contagious hospital, the exposed rooms were closed and placed under isolation technique. The tuberculous ward, where the first episode occurred, has an occupancy at all times of approximately 60 patients from six months to thirteen years of age; half of these patients are ambulatory. The medical ward, where the second episode occurred, has an occupancy of 75 to 80 patients, ranging in age from a few days to thirteen years; all are confined to bed. It is a striking observation that in each episode, in spite of the large number of children in contact with herpes zoster and the secondary eruptions resulting therefrom, only four cases designated as chickenpox occurred. In contrast with the lack of wide dissemination following exposure to herpes zoster and the resulting secondary eruptions, chickenpox epidemics are usually widespread in institutions following exposure to a single case. In these cases, as in chickenpox, the varicelliform eruption appeared from thirteen to twenty-one days after exposure to known cases of herpes zoster. This, together with the fact that three of the patients had been under observation in the hospital for more than a month with no visitors, would indicate that the relationship between herpes zoster and chickenpox is more than a mere coincidence.

#### HISTORICAL

Numerous cases of varicella following exposure to herpes zoster have been reported. French and British physicians have described many such incidences and a number have appeared in American literature.

Von Bokay<sup>1</sup> in 1892 in Budapest first called attention to the apparent relationship of these two diseases. In 1909<sup>2</sup> he reported further cases substantiating his original observation. He stated that children were most frequently



affected; and that the disease appeared usually after an incubation period of fourteen to twenty days, the accepted incubation period of chickenpox. He, together with Netter, contributed to the monistic theory which proposes that one virus causes both diseases, but that there occurs a mutation in the virus; first, in regard to its infectivity (high in varicella, low in herpes zoster) and secondly, in relation to its tissue affinity. In the case of herpes zoster the virus shows a pronounced neurotropism; in varicella, dermatropism. Comby,<sup>3</sup> on the other hand, adhered to a dualistic theory; he thought the two diseases were caused by different viruses.

Considerable experimental work has been carried on in an effort to establish the relationship between these two diseases. Netter and Urbain<sup>4, 5</sup> demonstrated by the complement fixation test that the sera of patients with herpes zoster and varicella contain identical antibodies. They used an antigen consisting of a saline extract of the crusts from the lesions. Moreover, they showed that antibodies to varicella are equally present in the serum of symptomatic zoster, such as follows ingestion of arsenic, mercury, or bismuth. In 1928 Netter<sup>6</sup> collected 174 examples of chickenpox contracted in this manner in France. He found the incubation period was from seven to twenty-four days, with more than half falling between thirteen and sixteen days. He also collected 25 cases of herpes zoster following chickenpox and found the same incubation period.

Lipschutz and Kundratitz<sup>7, 8</sup> inoculated infants with herpes zoster fluid and found that some developed local reactions and others developed a generalized varicelliform eruption. They also found that children in contact with positive reactors to the herpes zoster inoculations developed chickenpox. Further, they noted that children who had recovered from varicella proved immune to inoculations of herpes zoster fluid; and children who had recovered from natural or experimental herpes zoster were immune to varicella fluid. Finally, they found that serum of patients convalescing from herpes zoster could be administered as a protection against varicella, and that varicella and experimental herpes zoster had the same incubation period.

Kundratitz's experiments were repeated and confirmed by Bruusgaard,<sup>9</sup> although Lauda and Silberstern<sup>10</sup> did not agree with their results. Bruusgaard noted that the general exanthem following inoculation with zoster fluid was morphologically and histologically indistinguishable from varicella. Only children under five years of age proved susceptible. His cases simulated true varicella in every respect. He noted, however, that the fever was low, seldom over 100.4° F. to 101.3° F., and that the vesicles showed quick desiccation.

Paschen<sup>11</sup> reported the finding of elementary corpuscles similar to those of varicella and vaccinia in the blister fluid. Suspensions of these corpuscles were specifically agglutinated by the sera of herpes zoster convalescents. Amies,<sup>12</sup> following this work, showed that elementary bodies morphologically similar to those found in varicella were constantly present in vesicle fluid of herpes zoster. His attempts to demonstrate the relationship by cross-agglutination tests met with a fair measure of success.

Brain<sup>13</sup> confirmed the complement fixation experiments of Netter and Urbain, using antigen from herpes zoster and varicella vesicle fluid instead of of the crusts.

Goldberg and Frances<sup>14</sup> in 1918 reported three cases of simultaneous chickenpox and herpes zoster at the Cook County Hospital.

Goldsmith,<sup>16</sup> in reviewing the subject, stated that persons develop varicella following exposure to herpes zoster ten times more frequently than do persons develop herpes zoster following exposure to varicella. He also stated that Cantor,<sup>15, 16</sup> in 20 years' work on an island in the Pacific, saw many cases of herpes zoster but had never seen one case of chickenpox. Storen<sup>16</sup> in Norway had a similar experience; he had never seen varicella although he had seen cases of herpes zoster.

#### DISCUSSION

A review of the literature still leaves one in doubt as to whether the virus of the two diseases is identical or whether it is two separate entities. Varicella results in a more or less permanent immunity to itself. It is not unusual for individuals who have had varicella to contract herpes zoster subsequently, though herpes zoster is relatively an uncommon disease. In our experience chickenpox following exposure to herpes zoster has been infrequent. We have observed several epidemics of chickenpox in the Children's Division, and in each instance the number of individuals secondarily infected was numerous; it has been one of the most difficult contagious diseases to control. On the other hand, herpes zoster has appeared in a number of patients in the wards without being followed by other cases of herpes zoster or of chickenpox.

#### CONCLUSIONS

1. Two minor epidemics of chickenpox following herpes zoster are reported.
2. These episodes differ from the usual chickenpox epidemics in that so few secondary cases occurred among so many children exposed. It would seem that chickenpox follows exposure to herpes zoster in comparatively few instances. On the other hand, exposure to chickenpox has usually been followed by an epidemic in our institution.

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## ON THE PROBLEM OF DIARRHEAS OF CHILDHOOD: A STUDY OF 543 ATTACKS IN CHILDREN AT BEIRUT, SYRIA\*

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### INTRODUCTION

THE term "diarrhea" is used in this paper as meaning "abnormal frequency and liquidity of fecal discharges (Hippocrates),"<sup>1</sup> and without any connotation pertaining to etiology, severity, prognosis, or presence or absence of blood or mucus. It is now generally believed that diarrheas, which are characterized by blood, pus, and mucus in the stools, with accompanying toxemia, are usually due to infection with one or more species of micro-organisms whose pathogenicity has been established by conventional criteria. Diarrheas which are not characterized by the presence of blood in the stools and by toxemia have, however, been given less attention, and considerably less respect, because of their relative mildness and because it has frequently been impossible to isolate or identify any of the organisms which have been convicted as pathogens.

The series of cases discussed here represents typical sporadic diarrheas, about which little is known as compared with those which have been observed in epidemic form. The purpose of this paper is to present the results of a carefully made survey of the microbial flora and fauna, with consideration of the clinical aspects of the cases concerned. The laboratory data are presented as a basis for the clinical discussion and for such intrinsic value as a general knowledge of the organisms likely to be encountered in analogous cases may have. Between October, 1933, and June 30, 1936, 543 attacks of diarrhea which affected 490 patients were studied.† Many of the children under observation had more than one attack of diarrhea during the period of study, so that the number of attacks recorded is greater than the number of patients. These attacks were considered to be new illnesses rather than

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†The university hospital and clinics were closed from July 1 to October 1 for each of the years included in the study, and cases which occurred in the city during midsummer did not come within the scope of this study.

relapses, when the time between attacks was eight weeks or more; but most of the multiple attacks occurred at intervals of several months. Data pertaining to number of cases and number of attacks in relation to age and sex are given in Tables I and II.

TABLE I  
TOTAL CASES AND TOTAL NUMBER OF ATTACKS ACCORDING TO AGE GROUPS

	0-12 MO.	13-24 MO.	2-5 YR.	6-12 YR.	TOTAL
No. attacks	208	183	109	43	543
No. cases	193	160	95	42	490

TABLE II  
SEX DISTRIBUTION OF 543 ATTACKS

	0-12 MO.	13-24 MO.	2-5 YR.	6-12 YR.	TOTAL
Attacks					
Female	81	71	31	15	198
Male	127	112	78	28	345
Total	208	183	109	43	543
Cases					
Female	73	62	26	15	176
Male	119	98	69	27	313
Total	192	160	95	42	489

Approximately 50 per cent of the patients were not seen until after the fifth day of illness, and 11 per cent were not seen until after three or more weeks of illness. The patients examined by the laboratory were "unselected" in that no distinction was made on the basis of the type of diarrheal stool (i.e., bloody, mucous, or watery).

The seasonal incidence of the attacks studied is presented in Table III.

TABLE III  
SEASONAL INCIDENCE OF ATTACKS

	0-12 MO.	13-24 MO.	2-5 YR.	6-12 YR.	TOTAL
October	17	22	16	7	62
November	34	42	16	10	102
December	20	18	17	2	57
January	14	6	8	5	33
February	9	6	4	5	24
March	11	11	7	1	30
April	17	12	8	1	38
May	53	42	19	6	120
June	33	24	14	6	77
Total	208	183	109	43	543

#### METHODS OF LABORATORY EXAMINATION

Stools from hospitalized patients were sent directly to the laboratory in special containers. Patients from the Out-Patient Clinic were sent to the laboratory, where the stool specimens were obtained. Rectal swabs were used in the case of most infants. All stool specimens were examined at the laboratory as soon as received.

The specimen of diarrheal stool was routinely examined by both *bacteriologic and protozoologic methods*. The fresh specimen was streaked on bacto endo and bacto eosin-methylene blue agar plates. At the same time a portion of the specimen was emulsified in saline and examined for protozoa and for pus and blood cells. Helminth ova or larvae were reported when observed, but no special steps were taken to ascertain their presence. Two smears of each specimen were fixed in Schaudinn's fluid, stained with iron hematoxylin, and searched for protozoa.

The plates were incubated at 37° C. for twenty-four hours. The organisms present were subsequently identified by their action on differential media and by agglutination. The only bacterial species which was ignored was *Escherichia coli (communis)*. If an organism could not be immediately identified, it was maintained among our stock cultures until opportunity arose for further study. Organisms which gave characteristic sugar reaction, but which were not agglutinated by specific sera, were reported with reservations (i.e., *Salmonella*-like "species," and saved for further study).

The bacterial nomenclature followed is that of Bergey.<sup>2</sup>

#### RESULTS OF THE SURVEY

In Table IV are presented the number of times the various organisms were reported, their percentage incidence in the 543 attacks, and the number of times each organism was the sole finding except for *Escherichia coli*. The table also shows the distribution of the reports according to the ages of the patients. Most of the organisms reported occurred so infrequently that they cannot be evaluated, and are not given further consideration in this study.

Comparison of the data obtained in this survey with reports from other areas (Table V) is complicated by the fact that most reports are based upon a study of selected material, usually representing that phase of diarrhea designated as "dysentery." Reports on the systematic examination of stools of children for *both* bacteria and protozoa are extremely rare.

The incidence of *Giardia lamblia* (15.5 per cent) was higher than that of any other species of organisms reported, bacteria included, and was 25 per cent greater than that of the paradysentery group. Of particular interest is the age distribution (10 per cent in the first year, 15 per cent in the second year, 28 per cent in the 2 to 5 year group, and 9 per cent in the 6 to 12 year group) when compared with that of Boeck<sup>3</sup> for children who did not have diarrhea (0 in the first year, 7.4 per cent for the second year, 18 per cent for the 2 to 5 year group, and 15.8 per cent for the 6 to 12 year group). The incidence of *Giardia* in hospital patients in Beirut is 5.5 per cent,<sup>12</sup> and the incidence in children admitted for diseases other than diarrhea is only 7.5 per cent.

#### CLASSIFICATION OF DIARRHEA AND OBSERVATIONS IN RELATION TO APPEARANCE OF STOOL

In classifying the cases which have come under observation we have made an attempt to dissociate the purely descriptive aspects of types of diarrhea, based upon the appearance of the stool, from their clinical connotations, in

TABLE IV

INCIDENCE OF MICROORGANISMS ENCOUNTERED IN BACTERIOLOGIC AND PROTOZOOLOGIC EXAMINATIONS OF STOOLS OF 543 ATTACKS OF DIARRHEA

MICROORGANISM	0-12 MO.		13-24 MO.		2-5 YR.		6-12 YR.		TOTAL REPORTS		PER CENT INCIDENCE
	TOTAL	SOLE*	TOTAL	SOLE*	TOTAL	SOLE*	TOTAL	SOLE*	TOTAL	SOLE*	
<i>Shigella dysenteriae</i>	-	-	1	-	-	-	1	-	2	-	0.37
<i>Shigella ambigua</i>	-	-	2	2	-	-	-	-	2	2	0.37
<i>Shigella paradysenteriae</i>											
Var. Flexner	13	8	6	6	11	6	5	3	35	23	6.45
Var. Hiss Y	7	2	5	3	1	1	1	-	14	6	2.57
Var. Kruse E-Sonne	3	1	3	3	2	1	-	-	8	5	1.47
Var. Strong	1	-	3	3	-	-	-	-	4	3	0.74
<i>Salmonella paratyphi</i>	-	-	1	-	-	-	-	-	1	-	0.18
<i>Salmonella schottmuelleri</i>	-	-	1	-	-	-	-	-	1	-	0.18
<i>Salmonella hirschfeldii</i>	2	1	1	-	1	-	1	1	5	2	0.92
<i>Salmonella enteritidis</i>	-	-	3	2	1	1	2	-	6	3	1.10
<i>Salmonella morgani</i>	10	5	9	4	6	2	1	-	26	11	4.79
<i>Eberthella typhosa</i>	-	-	-	-	-	-	1	-	1	-	0.18
<i>Salmonella species</i> (?)	13	9	24	18	10	4	5	2	51	33	9.40
<i>Alcaligenes faecalis</i>	4	1	-	-	2	1	2	-	8	2	1.47
<i>Escherichia communior</i>	2	1	3	2	2	1	-	-	8	5	1.47
<i>Proteus vulgaris</i>	2	1	2	-	-	-	-	-	4	1	0.74
<i>Proteus asiaticus</i>	8	4	11	6	10	3	1	-	30	13	5.52
<i>Proteus asiaticus</i> var. syriensis†	1	1	3	1	-	-	-	-	4	2	0.74
<i>Klebsiella pneumoniae</i> (Friedländer's bacillus)	4	3	3	2	2	1	-	-	9	6	1.66
<i>Streptococcus viridans</i>	1	-	1	-	-	-	-	-	2	-	0.37
<i>Streptococcus faecalis</i>	21	18	14	9	4	2	4	1	41	30	7.55
Unidentified gram-negative bacilli	6	1	2	-	3	1	-	-	11	2	2.02
<i>Endamoeba histolytica</i>	-	-	2	1	9	1	6	3	17	5	3.23
<i>Endamoeba coli</i>	1	-	8	4	11	-	7	-	27	4	4.97
<i>Endolimax nana</i>	-	-	1	-	2	-	1	-	4	-	0.74
<i>Iodamoeba bütschlii</i>	1	-	2	2	-	-	1	-	4	2	0.74
<i>Diandamoeba fragilis</i>	-	-	-	-	-	-	1	-	1	-	0.15
<i>Giardia lamblia</i>	21	9	28	16	31	12	4	3	84	40	15.5
<i>Trichomonas hominis</i>	6	1	7	2	16	3	3	-	33	7	6.03
<i>Chilomastix mesnili</i>	7	5	5	3	14	1	3	-	29	9	5.35
<i>Blastocystis hominis</i>	1	1	2	-	1	-	1	1	5	2	0.92
Yeasts	2	1	2	2	2	-	1	1	7	4	1.29
<i>Trichuris trichiura</i>	1	-	-	-	1	-	-	-	2	-	0.37
<i>Ascaris</i>	3	1	1	-	3	-	2	1	10	2	1.84
<i>Taenia saginata</i>	-	-	-	-	3	1	6	1	9	2	1.66
<i>Hymenolepis nana</i>	-	-	1	-	10	-	4	-	15	-	2.76
Negative except for <i>Es. coli communis</i>	103		62		24		9		198		36.5

\*Columns headed "sole" indicate the number of times reported when the organism indicated was the only significant finding.

†An organism closely resembling *Proteus asiaticus* but showing certain cultural and serologic differences which are constant. To be fully described in a separate article.

TABLE V  
COMPARATIVE INCIDENCE OF ORGANISMS REPORTED IN DIARRHEA

MICROORGANISM	A. U. B. (BEIRUT)	ALI <sup>4</sup> (EGYPT)	DENSTED <sup>3</sup> (EGYPT)	DAVISON <sup>6</sup> (BALTIMORE AND BIRMINGHAM, U. S. A.)	DENNISON AND DE HALL <sup>7</sup> (ALABAMA, U. S. A.)	KHALED <sup>8</sup> (EGYPT)	WOLLSTEIN <sup>9</sup> (NEW YORK, U. S. A.)	HILL AND HILL <sup>10</sup> (PUERTO RICO)	HAYNES <sup>11</sup> (EGYPT)
<i>Shigella dysenteriae</i>	0.37	3.6	12.0	—	—	10.2	3.5	—	—
Paratyphoid group	11.23	18.45	46.0	42.5	74.5	56.3	19.8	—	45.0
Paratyphoid A, B, and C	2.38	—	0.3	—	—	2.8	1.5	—	—
<i>E. typhosa</i>	0.18	—	—	—	—	—	2.3	—	—
<i>S. morgani</i>	4.79	7.9	11.0	—	5.7	16.0	1.15	—	—
Late lactose fermenters and Salmonella-like organisms	9.4	—	—	—	48.5	—	2.3	—	—
<i>E. coli</i> communior	1.47	12.2	—	—	—	—	—	—	—
<i>Proteus asiaticus</i> group	6.26	—	—	—	—	2.8	—	—	—
<i>Proteus vulgaris</i>	0.74	0.7	—	—	22.8	—	—	—	—
<i>Alcaligenes faecalis</i>	1.47	—	2.7	—	14.3	—	1.15	—	—
<i>E. histolytica</i>	3.23	5.75	11.0	—	—	5.1	—	2.4	—
<i>Giardia lamblia</i>	15.5	—	—	—	—	—	—	47.2	—
Attacks	143	139*	300	71	35	215	86	125	40

\*Age group 0-24 mo.

TABLE VI  
PERCENTAGE INCIDENCE AND DISTRIBUTION OF SPECIES IN TYPES OF DIARRHEA

ORGANISM	PERCENTAGE INCIDENCE OF ORGANISMS			PERCENTAGE DISTRIBUTION OF ORGANISMS		
	BLOODY	MUCOUS	WATERY	BLOODY	MUCOUS	WATERY
<i>Shigella dysenteriae</i>	1	—	—	100	—	—
<i>Shigella ambigua</i>	—	0.8	—	—	100	—
Paratyphoid group	18	8	10	47	32	21
<i>Salmonella paratyphi</i>	—	0.4	—	—	100	—
<i>Sal. hirschfeldii</i>	0.6	—	3	20	—	80
<i>Sal. enteritidis</i>	2	1	—	50	50	—
<i>Eberthella typhosa</i>	—	—	0.7	—	—	100
<i>Salmonella</i> species (?)	7	13	5	23	63	14
<i>Alcaligenes faecalis</i>	3	0.8	0.7	63	25	12
<i>Escherichia communior</i>	2	0.8	2	37	25	37
<i>Proteus vulgaris</i>	1	0.8	—	50	50	—
<i>Proteus asiaticus</i>	5	7	4	26	57	17
<i>Proteus asiaticus</i> var. <i>syriensis</i>	1	0.8	—	50	50	—
<i>Streptococcus viridans</i>	—	0.4	0.7	—	50	50
<i>Strep. faecalis</i>	6	8	10	22	46	32
Unidentified	4	1	1	55	27	18
<i>Endamoeba histolytica</i>	7	0.8	2	71	11	18
<i>Endamoeba coli</i>	7	4	4	41	37	22
<i>Endolimax nana</i>	0.6	0.8	0.7	25	50	25
<i>Iodamoeba bütschlii</i>	—	0.8	1	—	50	50
<i>Diandamoeba fragilis</i>	—	—	0.7	—	—	100
<i>Giardia lamblia</i>	17	16	12	33	48	19
<i>Trichomonas hominis</i>	8	6	3	39	49	12
<i>Chilomastix mesnili</i>	6	4	7	34	31	34
<i>Blastocystis hominis</i>	1	0.4	1	40	20	40
Yeasts	3	0.4	1	57	14	29
<i>Trichuris trichiura</i>	1	—	—	100	—	—
<i>Ascaris</i>	2	2	0.7	30	60	10
<i>Taenia saginata</i>	2	0.8	2	45	22	33
<i>Hymenolepis nana</i>	6	2	1	60	27	13
Negative	27	35	48	22	45	33

order that the validity of these connotations might be tested. Nevertheless, the classification adopted necessarily tends to be parallel with the current medical, as well as the popular, conception, of 3 degrees of severity associated with 3 types of stools. On the basis of the appearance of a stool we have used the categories: (1) bloody diarrhea with mucus; (2) mucous diarrhea without blood, and (3) watery diarrhea without blood and without mucus.

The sex and age distribution of the patients were considered according to the descriptive types of diarrhea. In cases with blood in the stools the ratio of males to females was nearly 3 to 1, while in the cases of watery diarrhea the number of boys was almost the same as the number of girls.

The percentage incidence and distribution of the various reported species of microorganisms according to types of stools are shown in Table VI, an analysis of which suggests that there are factors other than demonstrable infection with recognized dysentery organisms that determine the presence of blood in stools.

The factor of age in relation to infestation with certain organisms is shown in Table VI, from which it appears that in infants under 2 years of age the probabilities are almost even that members of the paradysentery group, *Salmonella*-like (?) organisms, streptococci, or *Giardia* will be found in a diarrheal stool, but in those stools containing blood, the chances are two or three to one in favor of the occurrence of either a paradysentery species or *Giardia*. In preschool children the probabilities are about even that paradysentery bacilli, *Proteus asiaticus*, *Salmonella*-like organisms, *Endamoeba histolytica*, or *Giardia*, will be found in stools containing blood, while *Giardia* is found with greater frequency in the other two types of diarrhea. In school children diarrhea with blood is likely to show either paradysentery bacilli or *Endamoeba histolytica*, while in mucous and watery diarrhea *Salmonella*-like bacilli were found most frequently.

Apparently, insofar as diarrhea is concerned, age is one of the important factors which govern the reaction of a child to a particular organism as manifested by the type of stool and the severity of the illness. We find that the incidence of *Giardia* in bloody diarrhea of infants was 22 per cent (only 10 per cent in all infants); while in the school age group the incidence of *Giardia* in bloody diarrhea (9 per cent) was the same as the incidence in this age group, regardless of the type of diarrhea. There is a strong implication in these data that a factor of age susceptibility is operative in influencing the pathogenic role of *Giardia lamblia* infestation in infants.

*Duration.*—An attempt was made to determine whether there was any correlation between the type of diarrhea, the duration of illness, or the length of treatment, and the type of organism found in the stools. The average length of treatment, when certain organisms were the sole findings in the stools, showed very little variation; eight days for the paradysentery group; eight days for *Proteus asiaticus*; nine days for *Streptococcus faecalis*; and eight days for *Giardia lamblia*. However, when *Giardia* was found in combination with other organisms, the average length of treatment increased to thirteen days, a very great increase.



The mean duration of treatment of the entire group of patients according to age groups was as follows:

0-12 months:	16 days
13-24 months:	18 days
2-5 years:	21 days
6-12 years:	16 days

These figures include only those patients who were followed until they were known to be cured.

TABLE VII

PERCENTAGE INCIDENCE OF CERTAIN ORGANISMS IN AGE GROUPS AND TYPE OF DIARRHEA

	0-12 MO.	13-24 MO.	2-5 YR.	6-12 YR.
<i>a. In All Types of Diarrhea</i>				
Paradysentery group	12	11	13	14
<i>S. morgani</i>	5	5	5	2
<i>P. asiaticus</i>	4	6	9	2
<i>Salmonella</i> species	6	13	9	12
<i>Strep. faecalis</i>	10	8	4	9
<i>E. histolytica</i>	0	1	8	14
<i>Giardia lamblia</i>	10	15	27	9
<i>b. Bloody Diarrhea</i>				
Paradysentery group	24	16	13	17
<i>S. morgani</i>	7	6	3	4
<i>P. asiaticus</i>	2	5	10	4
<i>Salmonella</i> species	11	5	10	4
<i>Strep. faecalis</i>	9	5	3	4
<i>E. histolytica</i>	0	3	16	20
<i>Giardia lamblia</i>	22	16	19	9
<i>c. Mucous Diarrhea</i>				
Paradysentery group	8	5	15	0
<i>S. morgani</i>	5	6	6	0
<i>P. asiaticus</i>	4	10	10	0
<i>Salmonella</i> species	5	23	10	30
<i>Strep. faecalis</i>	9	8	6	10
<i>E. histolytica</i>	0	0	2	10
<i>Giardia lamblia</i>	6	19	37	0
<i>d. Watery Diarrhea</i>				
Paradysentery group	8	11	8	20
<i>S. morgani</i>	3	0	8	0
<i>P. asiaticus</i>	5	0	8	0
<i>Salmonella</i> species	3	5	8	10
<i>Strep. faecalis</i>	12	14	0	0
<i>E. histolytica</i>	0	0	10	0
<i>Giardia lamblia</i>	8	8	23	20

*Colic.*—One would expect that the child complaining of colic, with consequent disturbance of the household, would be more likely to receive early medical attention, but such was not the case. The incidence of colic in the series of cases, as a whole, is shown in Table VIII (A) as distributed among the different age groups and types of diarrhea. As will be seen from this table there is no significant degree of correlation between the manifestation of colic and the type of diarrhea.

The association of colic with the occurrence of reported organisms was significant in relation to only four of the species reported; other organisms reported in patients who had colic were too few to be evaluated. Table VIII

(B) shows the percentage association of colic with these four species or groups of organisms. It is particularly noteworthy that *Giardia lamblia* in the infant group shows such frequent association with colic, and that of the times *Giardia* was reported in cases of bloody diarrhea in the infant group, the incidence of colic was 90 per cent.

TABLE VIII

AGE GROUPS	(A)				(B)			
	PERCENTAGE INCIDENCE OF COLIC IN				PERCENTAGE ASSOCIATION OF COLIC WITH			
	TYPES OF DIARRHEA AND AGE GROUPS				DIFFERENT ORGANISMS			
	Bloody Diar- rhea	Mucous Diar- rhea	Watery Diar- rhea	Total	Para- dysentery Group	Strep. faecalis	E. histo- lytica	Giardia lamblia
0-12 mo.	65	19	19	29	35	19	0	57
13-24 mo.	57	25	8	34	35	36	50	32
2-5 yr.	55	31	31	38	40	25	60	32
6-12 yr.	83	90	40	75	86	50	60	50

In the 45 persons with dehydration observed there were 11 deaths, a rate of about 25 per cent; but this does not include a few persons who refused admission to the hospital and who probably survived for a brief period at home. Exclusive of 2 deaths, which were not attributable to diarrhea, there were 18 deaths in the entire series (7 males, 11 females). A single death was attributable to diarrhea alone, without dehydration; 7 deaths were due to dehydration with diarrhea; and 4 deaths were due to pneumonia, dehydration, and diarrhea. Pneumonia was the terminal event in the 8 persons in whom it occurred. Marasmus was a contributing factor in 4 persons.

Vomiting, which persisted beyond the day of onset of the diarrhea, appeared to be an important factor in the development of symptoms of intoxication.

Bacteriologic and parasitologic findings in the persons who died showed an incidence of organisms as follows: paradysentery group, 33 per cent; *Proteus asiaticus*, 11 per cent; *Salmonella*-like bacilli, 11 per cent; *Giardia lamblia*, 17 per cent; and negative, 44 per cent.

#### TREATMENT

On the whole, none of the drugs used showed any particular advantage over a purely dietary and hygienic method of treatment. Of course, definite indications for specific medications, such as the finding of *E. histolytica* in the stools, or severe colic, or the coincidence of malaria, were followed.

Every effort was made to have persons with dehydration admitted to the hospital, where fluids were immediately administered, and blood transfusions, in small quantities, were given when possible. Gastric lavage was performed as indicated; occasionally, this procedure was followed by marked benefit. Irrigation of the colon with starch solution, with sodium bicarbonate solution, or with some medicated liquid, was used in cases characterized by severe colic or great frequency of stool. No particular benefit was noted in favor of the medicated over the nonmedicated irrigations. No food was given these persons until the symptoms of intoxication began to be definitely improved, usually a matter of forty-eight to seventy-two hours. Water in very small quantities

was given, by teaspoon, at regular short intervals, even in the most intoxicated persons, but was discontinued if there was vomiting. As the symptoms of intoxication diminished, a modification of protein milk in small quantities was given at intervals of four hours. The amounts, as well as the concentration, were increased gradually as the child became able to tolerate more food.

The general routine of treatment of the majority of the persons who were not dehydrated was that used in most clinics. In the case of older children no cathartic was given, the child was immediately placed on the modified protein milk regime. This protein milk was used as the sole article of food for a period averaging about three days, when other low residue, high protein articles of diet were added.

The modification of protein milk was developed in this clinic about ten years ago, and has since been used with great convenience and success. It was originated with the idea of making some easily prepared high protein milk available for even the most ignorant mothers, and making it relatively inexpensive, so that it could be used by even the poorest. It is prepared as follows: One liter of thoroughly boiled and cooled milk is curdled by the addition of lemon juice (or other acid), strained or decanted, and the curd added to one liter of cultured milk. The latter, under the name of leban, is a universal food in this part of the world, but can be obtained from any Syrian restaurant in any part of the world. The leban is easily prepared by any housewife, who inoculates tepid, boiled milk with some of the previous day's leban, and surrounds the bowl with a blanket to retain overnight the heat generated by fermentation. By the next morning the milk is set in an exceedingly fine, smooth curd. The acid-precipitated curds are beaten up with the liter of leban to form a perfectly smooth liquid. Water may be added to this mixture as required, depending upon the needs of the individual baby. It is usually wise to add sugar in the proportion of 2 tablespoons to a liter, but the sugar should be added at the time of each feeding, otherwise it will become very unpalatable in a few hours. The caloric value of this mixture is about 70 or 75 per 100 c.c. It contains about 6 to 6.5 per cent protein; 3.5 to 4 per cent fat; and about 3 per cent sugar (mostly sucrose), depending upon the amount of fermentation which has taken place during culturing of the milk.

The effect of this protein milk on the stools of an infant or older child suffering from diarrhea is often very rapid, producing a formed, pale, pasty stool after about thirty-six to forty-eight hours. Often, however, mucus and occasionally blood will persist in the stools for some days after they have become formed. Colic and distention disappear with great rapidity, particularly in the case of older children, in whom the disappearance of colic has been almost magical. The food is palatable to most babies, and the use of lactic or hydrochloric acid as the medium for coagulating the milk seems to make it palatable to those who refuse the food prepared with lemon juice. Infants and older children may be kept on this food for months. The constipation resulting when the protein milk is used for a long period after an acute diarrhea is readily controlled by the addition of other foods in the diet, laxatives thus being rendered unnecessary. This modification of protein milk

appears to combine the beneficial effects of lactic acid milk with those of a high protein milk. The tolerance to fat seems to be rapidly regained, even in cases of long-standing chronic intestinal indigestion.

#### COMMENT

Diarrhea is encountered among the children of Beirut throughout the year, but the distribution of the cases is distinctly seasonal, with most of the cases occurring in November and in May. The seasonal incidence appears to be correlated with the seasonal incidence of flies.<sup>13</sup> Of the children afflicted, boys are seen at the clinic nearly twice as frequently as girls.

Parallel bacteriologic and protozoologic examination of diarrheal stools revealed the presence of *Shigella* and *Salmonella* bacilli which are commonly encountered in cases of diarrhea elsewhere, but they were not found as frequently as expected. *Shigella dysenteriae* infection is much less common than in Egypt, and amoebiasis of young children is exceedingly rare.

The evidence obtained indicates that the age of the child plays an important role in determining susceptibility and type of reaction to infection with *Giardia lamblia*, and to a smaller extent with Morgan's bacillus. *Giardia lamblia* in infants is capable of provoking a severe diarrhea which frequently becomes chronic. The frequency with which *Giardia* is encountered in the normal stools of adults cannot invalidate the cumulative positive evidence pertaining to the pathogenicity of this organism for infants and younger children. It is probable that the factors involved are nonspecific and pertain to individual susceptibility rather than to any properties of "virulence" inherent in *Giardia*, but the potentialities of this flagellate in the production of diarrhea of infants should be appreciated.<sup>14</sup> The evidence obtained in connection with Morgan's bacillus supports the concept of that organism as a pathogen.<sup>15</sup>

*Proteus asiaticus* has been reported by Khaled<sup>16</sup> as frequently causing an enteric-like fever in Egypt. This organism has never been isolated from the blood stream by our laboratory, but the present report indicates that it occurs with sufficient frequency in severe diarrhea to warrant further study. It is probable that this organism is widely distributed, but is usually ignored as a "Proteus" or slow-lactose fermenter. There can be little doubt that *P. asiaticus* is potentially pathogenic.<sup>17</sup>

Conceptions of "diarrhea" and "dysentery" should be reviewed from the point of view of classification. The present study indicates that diarrhea is a clinical entity of which "dysentery" and "simple diarrhea," with their usual connotations, are merely phases. In a broad survey there was no correlation between the species of etiologic agent and the type or severity of the diarrhea. On the contrary, if the effects of the neurotoxin of *Shigella dysenteriae* are excluded, the character of the clinical manifestations appears to depend on the reactivity of the intestine of the individual to irritation. It seems likely that organisms other than *Shigellas* and *Salmonellas* may, in a given child, provoke a response characteristic of so-called "dysentery." Of the attacks with which members of the genus *Shigella* were associated, 32 per cent were mucous diarrheas without blood; and 21 per cent were simple watery diarrheas, i.e.,

53 per cent of the incidence of dysentery bacilli were in cases which were not "dysentery." In the absence of competent laboratory examination any assumption as to the noninfectious nature of any case of diarrhea is unjustified and dangerous.

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# LABORATORY METHODS

## DETERMINATION OF ETHYL ETHER IN BLOOD\*

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THE procedure described herein is based upon a principle widely used in the determination of ether and alcohol in biological materials; namely, the quantitative oxidation of these substances to acetic acid by means of chromic acid. Nicloux,<sup>1</sup> and Shaffer and Ronzoni,<sup>2</sup> have applied this procedure to the determination of ether in blood. The present method contains a number of modifications which greatly facilitate the determination and increase the accuracy. It was used in a study of the effect of premedication on the ether concentration in surgical anesthesia in human subjects, the results of which are to be published soon.

### APPARATUS

A single unit of the apparatus is shown in Fig. 1. It is permanently mounted except for the 25 by 130 mm. tapered centrifuge tube (*A*) which contains the sample and the 150 c.c. fat extraction flask (*B*) which contains the chromic-sulfuric acid solution. The inlet and outlet tubes are provided with traps as shown. The vapors are quantitatively absorbed in the bead tower above the extraction flask (*3*). The tower (*C*) eliminates the use of a train of absorption tubes and possesses the further advantage of being easily cleaned. Air is passed through calcium chloride and concentrated sulfuric acid to free it of any volatile-interfering substances. It is drawn through the apparatus by means of suction applied at the outlet tube above the absorption tower.

Rubber stoppers and connections were used in the apparatus. An all-glass apparatus with ground glass connections probably would have improved the accuracy.

### REAGENTS

The precautions concerning the purity and method of storage of distilled water, noted by Friedemann and Klaas,<sup>4</sup> should be carefully observed.

Sodium tungstate. A 10 per cent solution.

Acid mercuric sulfate. Fifty-six cubic centimeters of concentrated sulfuric acid are added to 500 c.c. of water. One hundred grams of mercuric sulfate are added. The contents of the flask are heated until solution of the salt is complete. Sufficient water is then added to bring the volume to about 1,000 c.c.

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Sulfuric acid. A 75 per cent solution.

Potassium dichromate. Approximately 0.1 N solution.

Sodium thiosulfate. An approximately 0.1 N solution is prepared and standardized with standard potassium iodate solution. An exactly 0.01 N solution is prepared each day by dilution. This solution may be used on several succeeding days; however, the daily preparation of a fresh solution is recommended.

Potassium iodide, C.P. Crystals finely ground.

Soluble starch solution. Approximately 5 per cent solution.

#### PROCEDURE

Before the determination exactly 5.0 c.c. of 0.1 N potassium dichromate are pipetted into the 150 c.c. extraction flask, approximately 25 c.c. of 75 per cent sulfuric acid are added, and the flask is connected to the tower. Approximately 5 c.c. each of acid-mercuric sulfate and sodium tungstate reagents are placed in the tapered centrifuge tube. The latter is provided with a cork stopper and is kept in the refrigerator until needed.

The skin is sterilized with mercury bichloride solution. The blood is drawn past the mark into the 2 c.c. precision syringe (made by Becton, Dickinson and Co., Rutherford, N. J.) which contains a few crystals of sodium oxalate. The syringe is held vertically while the blue plunger is slowly moved upward to the 2.0 c.c. mark. Excess blood is absorbed in a gauze sponge. The needle is then plunged to the bottom of the centrifuge tube, below the cold reagents, and the syringe is slowly emptied. The stopper is replaced and the sample is taken to the laboratory for immediate analysis.

The stopper is inserted into the top of the absorption tower; the suction is properly adjusted. The tube containing the sample is connected to the apparatus. The sample tube is gradually heated with a microburner flame until condensation appears in the Kjeldahl trap above. Heating of the sample is now discontinued. The trap is gently warmed to insure quantitative transfer of the ether vapors. The entire period of heating and aeration is about five minutes.

The sample tube is now disconnected. The stopper is removed from the top of the tower, and approximately 10 c.c. of distilled water are poured through the tower. The flask is heated rather rapidly for fifteen to twenty seconds (until vapors arise) and is allowed to stand about one minute. It is detached and lowered to the shelf; three to four additional rinsings of 10 c.c. each of water serve to wash the last traces of the reagents from the tower into the flask below. The flask is then placed in a cooling bath of running water or ice.

After each determination approximately 100 c.c. of 75 per cent sulfuric acid are poured through the aeration tower to remove the remaining water. This solution may be used repeatedly until diluted. Any precipitate adherent to the inlet tube is washed and wiped off. The apparatus is now ready for another determination.

The residual oxidizing agent is determined iodometrically. Approximately 0.5 Gm. of potassium iodide is added. One-hundredth normal sodium thiosulfate is added until most of the color has disappeared. Starch is then added, and the titration is continued until the solution is colorless.

Blank determinations are made simultaneously with the reagents. The difference between the cubic centimeters of sodium thiosulfate required by the blank and that required in the titration of the sample represents the cubic centimeters of sodium thiosulfate equivalent to the oxidizing agent reduced by the ether. One cubic centimeter of 0.01 N sodium thiosulfate is equivalent to 0.0926 mg. of ether. This factor corresponds to the quantitative oxidation of the ether to acetic acid.

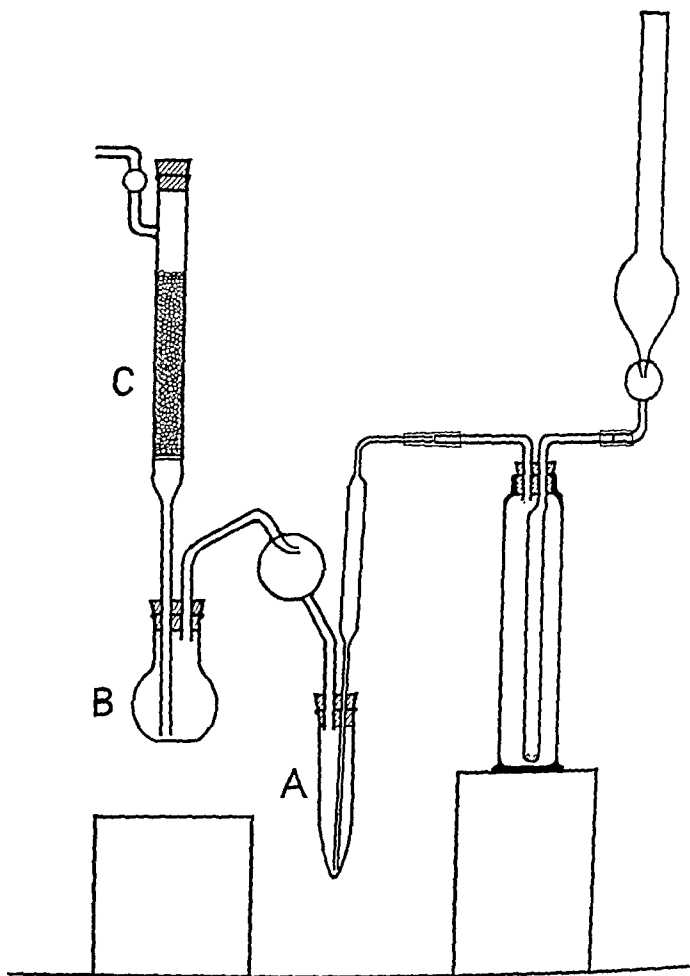


Fig. 1.

## DISCUSSION

In addition to describing a simple and convenient form of apparatus, we wish to call attention to the use of syringes for the accurate measurement of small samples. The customary collection of samples in tube and the subsequent measurement by means of pipettes may involve large losses of volatile substances. The direct measurement of the sample from the syringe into the tube or reaction flask has not been heretofore suggested as far as we are aware. The accuracy of precision syringes for this purpose may be judged by the following data.



Seven syringes were fitted with No. 18 hypodermic needles. Duplicate deliveries of 0.100 N hydrochloric acid from the syringes required 20.39, 20.37, 20.16, 20.14, 20.10, 20.10; 20.35, 20.26; 20.30, 20.24, 20.40, 20.40; and 20.10 20.20 c.c. for neutralization to phenolphthalein with 0.01 N sodium hydroxide. Single titrations of 0.100 N hydrochloric acid delivered by 2.0 c.c. pipettes required 19.96, 20.01, 20.02, 19.84 c.c. of 0.01 N sodium hydroxide. The syringes delivered only from 0.5 to 2 per cent more of solution than the pipettes. The close agreement of the duplicate samples should be noted.

TABLE I  
RECOVERY OF ETHER FROM SOLUTION

WT. OF ETHER	PURE SOLUTION		PURE SOLUTION PLUS BLOOD AND REAGENTS	
	THIOSULFATE TITRATION	RECOVERY	THIOSULFATE TITRATION	RECOVERY
mg.	c.c. 0.01 N	Per cent	c.c. 0.01 N	Per cent
1.042	11.0	97.7	11.1 10.9	98.6
	11.2	99.4		97.0
	11.1	98.6		
1.256	13.4	98.6	16.8 17.0	98.6 100.4
	13.6	100.2		
1.570	16.8	98.6		
	16.7	98.4		
1.884	20.0	98.4		
	20.5	100.6		
	20.2	99.4		
	20.2	99.4		
2.198	23.7	99.8		
	23.8	100.3		
	23.4	98.6		
	23.2	97.9		
2.512	27.4	101.0		
	27.2	100.4		
	27.0	99.5		
	27.1	99.8		
2.826	30.7	100.5		
	30.2	98.9		
	30.4	99.4		
	30.3	99.3		
3.140	34.0	100.3		
	33.8	99.6		

The recovery of ether from pure solution is shown in Table I. Data are given also in the table for the recovery of ether from pure solution in which have been added the precipitating solutions and 2.0 c.c. of fresh whole blood. The quantities of ether indicated in the table are within the concentrations found in animals in ether anesthesia. Considering the volatility of ether, the procedure yields satisfactory results.

#### SUMMARY

The use of precision syringes for accurate measurement of blood samples that may contain very volatile substances is described. The sample is delivered below the surface of cold precipitating reagents, acid mercuric sulfate-sodium

tungstate, from which the ether is removed by rapid aeration with heating. The vapors are quantitatively absorbed in a bead tower containing chromic sulfuric acid mixture. The residual oxidizing agent is determined iodometrically.

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## THE PHOTOELECTRIC STANDARDIZATION OF POTASSIUM PERMANGANATE SOLUTIONS\*

WILLIAM S. HOFFMAN, PH.D., M.D., CHICAGO, ILL.

**P**ERMANGANATE solutions are notoriously difficult to keep standardized because they deteriorate rapidly. For accurate analyses, they must be restandardized at intervals not greater than two weeks. This is usually accomplished by having on hand a standard solution of sodium oxalate, which apparently can be kept unchanged for several months. A safer but more tedious procedure is to weigh out each time a definite amount of sodium oxalate, dissolve it in approximately normal sulfuric acid and titrate it at about 70° C. with the permanganate solution. Even this procedure can be inaccurate because the deteriorated permanganate solution has varying quantities of precipitated oxides of manganese (presumably  $MnO_2$ ) which may be stirred up and included in the titration.

This difficulty in maintaining standard solutions of permanganate is felt particularly in biological laboratories which use such solutions only occasionally in the determination of serum calcium. Often the failure on the part of the technician to take the trouble to restandardize the permanganate solution makes the serum calcium determination worthless.

The author has found that, with the aid of the photoelectric colorimeter, the standardization of potassium permanganate can be made in a few minutes, with an error of less than 1 per cent. The procedure is based on the fact that the readings of varying dilutions of the permanganate in the photoelectric colorimeter with a green filter, when plotted on semilogarithmic paper, make a smooth curve, which for narrow ranges is partially a straight line.

The standard curve is prepared by diluting 4, 5, and 6 c.c., respectively, of accurately standardized 0.05 N potassium permanganate to 200 c.c. in volumetric flasks. These solutions, which are N/800 with factors of 0.800, 1.000, and

\*From the Department of Physiological Chemistry, Chicago, Medical School.  
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1.200, respectively, are read in the photoelectric colorimeter\* with a green filter, with maximum transmission at  $530\text{ m}\mu$  (Cenco filter No. 2). The values are plotted on semilogarithmic paper as in Fig. 1.

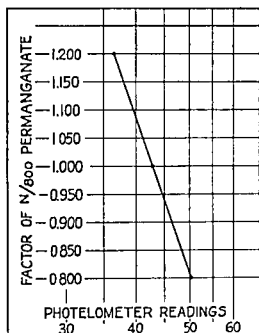


Fig. 1.—The relationship between photometer readings and concentration of potassium permanganate. The ordinate is expressed as the factor by which  $N/800$  potassium permanganate must be multiplied to give its concentration.

The permanent curve is used to standardize extemporaneous solutions prepared from one concentrated stock solution. This preparation is best illustrated by an example. The stock solution was approximately 1.0 normal. A 0.01  $N$  solution was desired. Five cubic centimeters of the stock solution was diluted to 500 c.c. in a volumetric flask. Twenty-five cubic centimeters of this approximately 0.01  $N$  solution was diluted to 200 c.c. in a volumetric flask and read in the photoelectric colorimeter. The reading was 44.0, which meant that the 0.01  $N$  potassium permanganate had a factor of 0.965. The solution could be discarded at the end of the day, for it required only a few minutes to prepare another standardized solution.

#### SUMMARY

A photoelectric method for standardizing potassium permanganate solutions is described. It is simple, rapid, and accurate.

\*The Cenco-Sheard-Sanford photometer, manufactured by the Central Scientific Co., was used in these experiments.

## A QUANTITATIVE STUDY OF SYPHILITIC SERUM\*

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CHARLES C. CROFT, M.S., TUCSON, ARIZ.

THERE has been much discussion as to whether the results of laboratory tests for syphilis should be expressed as negative, doubtful, or positive, or in terms of plus. The former method does not give much information as to the effect of treatment, and the latter method may lead to difficulties, for, as Eagle<sup>1</sup> has pointed out, laboratory tests for syphilis, as usually performed, are not quantitative. Results reported in terms of plus often fail to give information regarding the effect of treatment, since the tests, as usually performed, have a maximum limit of four-plus in undiluted serum, and quantities of reagin in excess of that required to give a four-plus (i.e., complete) reaction are not shown in the result. Wiener,<sup>2</sup> in a quantitative study of the Kline test, found that some sera would give positive reactions when diluted one thousand times.

Several investigators<sup>1-3</sup> have discussed the desirability of expressing results on a quantitative basis. Quantitative procedures have been described for complement fixation tests and for flocculation tests. Our investigation was made to determine the quantities of reagin which might be present in syphilitic sera, and the possible application of quantitative methods as a routine procedure for following the effects of treatment.

### PROCEDURE

When a serum gave a positive laboratory test for syphilis, it was diluted with physiologic saline, and the Hinton and Kline diagnostic tests were performed on the diluted serum in the usual manner. The results were recorded as negative, doubtful, or positive. At the same time a series of positive sera were examined, an equal number of negative sera were treated in a like manner. These negative sera, without exception, gave negative reactions in all dilutions. The results are reported in terms of *serum dilutions*. In the Hinton test the final dilutions of the serum-antigen mixture are actually twice those given (0.5 c.c. indicator and 0.5 serum are employed in the tests).

The maximum serum dilutions in which positive reactions were noted are shown in Table I.

Table II gives the relation of positive tests on diluted sera (from treated and untreated patients) to the Kahn reaction on the same sera undiluted.

Table III shows the maximum dilution of sera of known treated cases in relation to the Kahn test on the same sera undiluted.

\*From the Arizona State Laboratories, Tucson and Phoenix.  
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TABLE I  
MAXIMUM DILUTIONS OF SERUM WHICH GAVE POSITIVE REACTIONS

TEST	NUMBER OF SERA								TOTAL
	UNDILUTED	1:1	1:2	1:4	1:8	1:16	1:32	1:64	
Hinton*	1	16	22	18	24	14	6	5	106
Kline*	7	23	27	11	22	3	10	3	106
Hinton only	1	2	6	3	3	5	1	0	21

\*Performed on the same sera.

TABLE II  
MAXIMUM TITERS OF SERA FROM ALL PATIENTS (TREATED AND UNTREATED) IN RELATION TO THE KAHN TEST

KAHN (UNDILUTED SERUM)	TEST	NUMBER OF SERA								TOTAL
		UNDILUTED	1:1	1:2	1:4	1:8	1:16	1:32	1:64	
1 plus	Hinton	0	2	5	0	0	0	0	0	7
	Kline	3	4	0	0	0	0	0	0	7
2 plus	Hinton	1	4	6	1	4	1	0	1	18
	Kline	3	8	3	1	2	0	1	0	18
3 plus	Hinton	0	5	8	8	3	0	1	0	25
	Kline	1	11	9	1	1	0	2	0	25
4 plus	Hinton	0	0	0	6	12	11	7	5	41
	Kline	0	1	9	8	11	3	6	3	41

TABLE III  
MAXIMUM TITERS OF SERA FROM TREATED PATIENTS IN RELATION TO THE KAHN TEST

KAHN (UNDILUTED SERUM)	TEST	NUMBER OF SERA					TOTAL
		1:1	1:2	1:4	1:8	1:16	
1 plus	Hinton	0	4	0	0	0	4
	Kline	4	0	0	0	0	4
2 plus	Hinton	0	4	1	0	0	5
	Kline	5	0	0	0	0	5
3 plus	Hinton	1	3	2	0	0	6
	Kline	5	1	0	0	0	6
4 plus	Hinton	0	1	4	2	1	8
	Kline	4	3	0	1	0	8

#### DISCUSSION

An examination of Table II reveals that the arbitrary designation of results in terms of plus does not always give a true picture of the actual reagin content of sera. In the one-plus Kahn groups, all the sera gave positive Hinton reactions when diluted (2 in 1:1, and 5 in 1:2); the same relationship holds in the sera of higher reagin content, but it will be noted that in the three-plus Kahn group, 13 (approximately 50 per cent) gave positive Hinton reactions in maximum dilutions of 1:1 or 1:2, and 21 (82 per cent) gave positive Kline reactions in the same maximum dilutions. The limitation of the upper limit of four plus is well illustrated by the fact that in this group 100 per cent gave positive Hinton tests in dilutions of 1:4 or greater; 60 per cent in 1:16 or greater; and 30 per cent in 1:32 and 1:64.

Unfortunately, we have no detailed records regarding the patients whose sera are given in Table III. Judging from our experience, we would assume

that treatment had brought about a decrease in titer. In general, the maximum titers in Table III occur in lower dilutions than those of Table II, which includes serum from treated and untreated patients.

We feel that the Hinton or Kline test, quantitatively applied, gives valuable information as to the reagin content of serum, and should be particularly valuable in eliminating zone reactions which are sometimes encountered, particularly in tests where a single serum : antigen ratio is employed.<sup>4</sup>

Subsequent studies have shown that the Mazzini test may be readily performed quantitatively.

#### CONCLUSION

The quantitative application of the Hinton and Kline tests is described and discussed.

A quantitative examination eliminates zone reactions and gives a better index to the effect of treatment than the results expressed in the conventional manner.

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## A STUDY OF THE DEMONSTRATION OF TUBERCLE BACILLI IN URINE BY MEANS OF PHOSPHATE FLOCCULATION\*

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**I**N PRECEDING studies it was learned that direct centrifugation is an ineffective means of collecting tubercle bacilli from sputum,<sup>1</sup> spinal fluid,<sup>2</sup> and urine.<sup>3</sup> In urine containing both typhoid and tubercle bacilli, centrifugation for thirty minutes was found to collect 99 per cent of the typhoid bacilli and only about 20 per cent of the tubercle bacilli (unpublished data). During the present study of the concentration of tubercle bacilli from urine, an analysis of the Petroff "tannic acid" procedure<sup>4</sup> showed that the precipitates produced by acidification and chilling of urine (with or without tannic acid), though bulky, gave better concentration than direct centrifugation. These precipitates contained approximately 70 per cent of the bacilli from the urine samples, whereas the final concentrates, after dissolution of the primary precipitates, contained roughly 1 per cent of the bacilli. It was recommended,<sup>3</sup> therefore, that the first precipitates be used without further attempts to reduce their volume

\*From the Department of Bacteriology, Hygiene and Preventive Medicine, School of Medicine, the George Washington University.

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by procedures involving direct centrifugation. It may be seen that direct centrifugation was unsatisfactory for collecting the bacilli from the original specimens or for washing the sediments collected by other methods.

It is the purpose of this communication to present more complete information on the collection of tubercle bacilli from urine and to deal especially with the following questions: (a) the degree of concentration obtained in urine by direct centrifugation in two types of centrifuges; (b) the efficiency of collection and concentration by several flocculation methods; (c) the factors which influence the results obtained by flocculation; and (d) the problem created by the presence of pus in clinical specimens. Such studies necessitated the use of known numbers of clump-free tubercle bacilli in normal urines. Insofar as it was possible, the methods were analyzed to obtain a measure of the practical degree of concentration for microscopic purposes (concentration factor) and the percentage of the total bacilli in the urines which were collected in the precipitate (percentage efficiency), which is important for cultivation or animal inoculation.

#### METHODS

Details concerning the preparation, preservation, and counting of clump-free suspensions of human tubercle bacilli have been given.<sup>2</sup> Known amounts of these precounted suspensions were added to measured volumes of urine from normal persons in order to provide experimental materials containing known and appropriate numbers of tubercle bacilli per cubic centimeter. The drained precipitates or sediments collected by various means were suspended in an equal volume of the alum-milk fixative, and 0.02 c.c. was spread on square slide areas of 2 by 2 cm. Counting, as before, was along the diagonals of the films.<sup>2, 5</sup> Ten fields were observed per diagonal, making 40 fields for the two films on each slide. From knowledge of the number of bacilli added to each cubic centimeter of urine, the average of bacilli to be expected per microscopic field in the unconcentrated urine was calculated and regarded as unity. The average number of bacilli per field produced by the application of a given concentration method represented a multiple of the unit number and indicated the *concentration factor*.

By measuring the volume of sediment collected from a given volume of urine, the "volume reduction" and the collecting efficiency of the methods were calculated. For example, if the volume of a sample was reduced 100 times as the result of concentration, the bacterial count per unit slide area should be increased 100 times if all the bacilli had been collected. The following formula permitted calculation of the *percentage efficiency*:

$$\frac{\text{Bacilli per field in concentrates}}{\text{Bacilli per field in urine} \times \text{volume reduction}} = \text{Percentage efficiency}$$

#### EXPERIMENTAL RESULTS

Alum and ferrie chloride, the flocculating agents which had been used to collect tubercle bacilli from sputum, could not be used in urine. Their flocculation was inhibited in varying degrees by different urines, so that a suitable standard dose of reagent could not be selected. The various salts and ions in

urine itself offered several possibilities for the formation of floccular precipitates. Two such precipitates were studied. Acidification and chilling of the urine produced precipitation without the addition of tannic acid. Because of the microscopic appearance of the precipitates and the conditions under which urates are known to become insoluble, these sediments were called "urate" precipitates. The flocculations which resulted from neutralization or slight alkalization of urine (constant shaking) were referred to as "phosphate" precipitates.

After preliminary study of direct centrifugation, "angle" centrifugation, phosphate precipitation, Petroff's tannic acid method,<sup>4\*</sup> and urate precipitation, four experiments on urines containing differing numbers of tubercle bacilli were carried out in order to make comparisons. The results summarized in Table I show the *concentration factors* for all methods, and the *percentage*

TABLE I

CONCENTRATION FACTORS AND PERCENTAGE EFFICIENCIES OF SEVERAL METHODS OF COLLECTING TUBERCLE BACILLI FROM URINE

EXPER. NO.	DIRECT CENTRIFUGATION		ANGLE CENTRIFUGATION		PHOSPHATE FLOC. (MINIMAL)		URATE PRECIPITATION		PETROFF 1ST STAGE	
	C. F.*	EFF. %*	C. F.	EFF. %	C. F.	EFF. %	C. F.	EFF. %	C. F.	EFF. %
1	30	—	10	—	164	80	57	78	87	54
2	81	—	73	—	128	75	77	40	41	92
3	60	—	50	—	100	52	59	92	77	47
4	31	—	23	—	143	79	85	29	81	59
Av.	50	?	39	?	134	71	70	60	71	63

\*Concentration factors (C.F.) and percentage efficiency (Eff.). The amount of sediment obtained by centrifugation did not permit determination of the efficiencies of these methods.

efficiencies of the flocculation procedures. Since the concentration factors from horizontal centrifugation at RCF. 500 or 1,000  $\times$  gravity for one hour were relatively low, and the volumes of sediment were too small for measurement (reason for absence of percentage efficiencies), the ineffectiveness of this method is apparent. The flocculation methods produced from 50 to 150 times more sediment, which contained a greater number of bacilli per unit volume. The difficulty with direct, horizontal centrifugation was not overcome by the use of greater centrifugal forces in a more efficient machine of the angle type. Although more bacilli were collected in the angle centrifuge, they were packed in clumps. Since clumps influenced the possibility of diagnosis only by virtue of their numbers, estimation of the number of bacilli per clump was useless and these clumps were recorded as one bacillus.

Comparisons between the three flocculation methods indicated that the use of tannic acid in Petroff's procedure was unnecessary and that the acidification and chilling of the urines produced comparable results. The minimal phosphate flocculation produced higher concentration factors than the urate precipitates and was slightly superior in percentage efficiency. Since the results

\*Instructions for this method differ in various sources where it is quoted. The method employed was presented to one of us (J. H. H.) by Dr. Petroff. It was followed strictly, except that the recommended centrifuging time was extended to ten minutes in order to pack the sediments more tightly.



shown for the flocculation methods were obtained by centrifuging for only five or ten minutes, direct centrifugation was not studied further.

The advantages of the phosphate over the urate precipitates may be summarized as follows: relatively constant amounts of calcium, magnesium, and phosphate ions are excreted daily in the urine, while the output of urates is influenced to a greater degree by diet. The urate precipitation required not only addition of acid but also variable periods of chilling, while the volume of precipitate was usually large and could not be regulated successfully. The phosphate flocculation appeared promptly after adjusting the pH of urine, and the amount of precipitate could be controlled within reasonable limits. Since the collecting efficiency of the phosphate flocculations on the average was slightly higher, and since the degree of concentration by any method cannot exceed the ratio between the original volume of urine and the final volume of sediment (volume reduction), the phosphate flocculation method was chosen for further study.

The relationship between the pH to which the urine was adjusted (to produce different volumes of precipitate) and the concentration factors and percentage efficiencies was investigated. Four pH levels were used as the basis of comparison; these were pH 6.6 to 6.8 (the approximate pH range of the minimal phosphate flocculations previously mentioned), pH 7.0, pH 7.5, and pH 8.0. Differing numbers of bacilli, capable of producing accurate counts after concentration, were added to clear, filtered urines. These large samples were divided into four portions of 40 to 200 c.c. in the different experiments. The pH of each portion was adjusted by the addition of N/1 or 2.5 N sodium hydroxide during constant shaking of the samples. The minimal flocculation point was used without regard to the pH at which it occurred, while the remaining three portions were adjusted to their respective pH values in the presence of phenol red indicator and color controls. As before the volume of precipitate collected by centrifugation was measured as carefully as possible in pipettes. After the smears had been counted, the concentration factors and the percentage efficiencies were calculated.

The results of four experiments showed clearly the necessity of producing small amounts of precipitate in order to obtain suitable concentration factors.

The highest concentration factors, average 124, were obtained from the minimal flocculations; the values fell progressively with increasing amounts of precipitate to an average factor of 56 at pH 8.0. The average percentage efficiencies indicated that in these experiments approximately 64 per cent of the bacilli were collected by minimal flocculations, while 83 per cent were collected at pH 8.0. The concentration factors and the percentage efficiencies at pH 7.0 and 7.5 were intermediate.

These experiments revealed much more comparable results from experiment to experiment in the minimal flocculations (pH not restricted to a given point) than in the portions of urine adjusted to the pH values designated. This result appeared to be explained by the fact that the different urines gave different amounts of precipitate at the given pH, possibly because of differing contents of the ions to be precipitated. Since the increased precipitation at higher pH

values interfered with concentration factors much more than it improved the completeness of collection, it became apparent that large volumes of urine and minimal phosphate flocculation provided the best combination of conditions.

Considerable attention was given to the possibility of double stage concentrations as a means of further increasing the volume reduction and the possibility of detecting small numbers of tubercle bacilli. The phosphate precipitates, collected as described at approximately neutral pH and termed primary precipitates, were dissolved in an equal volume of 12 per cent sulfuric acid (by volume). After incubation of the acid mixture at 37° C. for thirty minutes (if for cultivation), the solution was adjusted to grass green (pH about 4.5) in the presence of bromocresol green color indicator. A new, smaller precipitate (called secondary or second-stage precipitate) formed and was collected by brief centrifugation.

TABLE II

COMPARISON OF THE CONCENTRATION FACTORS IN PRIMARY AND IN DOUBLE-STAGE PHOSPHATE PRECIPITATES

EXPER. NO.	CONDITIONS OF PRIMARY PRECIPITATION	PRIMARY CONCENTRATION FACTORS	DOUBLE-STAGE CONCENTRATION FACTORS
1	pH 7.5*	×56	×106
2	pH 7.5	×60	×200
3	pH 7.5	×66	×254
4	pH 7.5	×51	×220
5	pH 7.0	×82	×358
6	Minimal flocculation	×164	×236
Averages		×80	×246

\*It has been shown that higher concentration factors are obtained at lower pH values.

Table II shows a comparison of the concentration factors obtained after single- and double-stage concentration of a series of urines containing known numbers of tubercle bacilli. Since most of these experiments were carried out before the importance of minimal flocculation was understood, the values are probably lower than those obtainable at the present time. However, the results indicate that it was possible to increase the primary degree of concentration by about three times, and that this double-stage procedure served the very important function of recollecting the bacilli after precipitates were dissolved in acid to kill contaminants.

During the course of the preceding experiments it was possible to procure for practical trial of the methods studied only 17 specimens of urine from 13 persons suspected of having renal tuberculosis. In view of the greater uncertainty of effective concentration in urines containing pus, these specimens included as many purulent samples as could be obtained. It was impossible to determine how many of these urines were from kidney disorders other than renal tuberculosis.

Each urine was placed in a large flask that permitted adequate shaking during adjustment of the pH to induce a slight phosphate flocculation. The urine was then poured into a large (open or separatory) funnel in which the precipitate settled. The sediment was drawn off into a 50 c.c. centrifuge tube as it collected. The tube of sediment was centrifuged, the supernatant fluid

was discarded, and more sediment was added for packing. Although this procedure did not permit collection of the last traces of precipitate, it removed the necessity of centrifuging large volumes of urine. After concentration by one or more methods the same number of microscopic fields (usually 50 to 100) was examined in each concentrate.

Among the four clear specimens of urine, two were positive for acid-fast bacteria. One of these was microscopically negative in the sediments from four concentration methods; positive cultures were obtained from the secondary phosphate precipitate. The other was microscopically positive in the second-stage phosphate flocculation, and negative by two other concentration methods.

Among the 13 purulent urines, three were shown to be microscopically positive for acid-fast bacteria. Only one contained large numbers of bacilli. The pus collected by direct centrifugation was strongly positive on microscopic examination. This urine was then fractionated in order to learn whether the preponderance of bacilli occurred in the pus or in the fluid urine. Bacilli in the clarified fraction of the urine were rare, being found only in the portions which were concentrated by primary and by secondary phosphate flocculations. The results from three other concentration methods were negative. It appeared that purulent sediments could not be discarded in order to avoid the difficulties which they caused. One of the remaining positive urines yielded two bacilli per 100 microscopic fields following direct centrifugation, and 15 bacilli per 100 fields following the second-stage phosphate flocculation. The last urine (very bloody) was negative in the first-stage phosphate precipitate, but positive after carrying out a second-stage procedure in which chloroform was used as the collecting agent.

In urines containing pus the greatest difficulty was found to be the limited possibility of obtaining suitable volume reduction, without which effective concentration was impossible. The bulky precipitates, obtained in the presence of minimal phosphate flocculations, were treated in a variety of ways designed to digest the pus prior to a second flocculation to collect the bacilli in smaller volume. Although these attempts accounted largely for the positive results which were obtained, they were not highly satisfactory. Acids dissolved the phosphate and not the cells, while strong alkali dissolved the cells but not the phosphate. Approximately an equal volume of 2.5 N sodium hydroxide was required to bring about appreciable solution of the cells. This combination formed a thick, gelatinous mass. The subsequent dilution and neutralization of these mixtures, together with the poor volume reduction in the presence of the residual cell debris, did not permit achievement of greatly increased concentration.

From primary phosphate precipitates treated with acid or with alkali, collection of the bacilli with xylol or chloroform was compared with the results obtained by double-stage phosphate flocculation. Although xylol flotation appeared more hopeful as a means of separating the bacilli from the insoluble residues, the degree of concentration was no better than in the bulky debris collected by the double-stage phosphate method. The results with chloroform likewise were not satisfactory, perhaps because this reagent was collected in the bottom of the tubes along with the undissolved residues.

## DISCUSSION

From the studies in the synthetic tuberculous urines it became clear that, among the methods studied, the precipitation of a very small amount of phosphate from urine by partial neutralization afforded the most practical and effective method of collecting tubercle bacilli. Because of the differing amounts of precipitate produced at an accurately controlled pH, exact control of this condition was less useful than the adjustment of the urine to the minimal flocculation point.

Earlier studies<sup>3</sup> had shown that urate or "tannic acid" precipitates could not be dissolved or washed by Petroff's method of direct centrifugation without losing the majority of the bacilli collected. In the case of the phosphate flocculation method, this objection to dissolving precipitates was overcome in a procedure that employed reprecipitation of phosphate as a means of recollecting the bacilli and increasing the degree of concentration. Thus it became possible to treat primary phosphate precipitates with acids prior to microscopic examination or cultivation.

Two factors limited the second-stage concentration: (a) the volume reduction was small compared with that obtained in the first stage; (b) the first precipitates were diluted four or five times during the process of dissolving in acid and then neutralizing the solution. Thus secondary stage increases of three times over the primary concentration of the bacilli were actually concentrations of twelve to fifteen times when credit is given for overcoming the effects of dilution. In no case did the phosphate double stage result in loss of concentration.

## SUMMARY

Among the five methods studied, the most effective means of collecting tubercle bacilli from urine was by partial neutralization to obtain a minimal phosphate flocculation. The factors influencing the degree of concentration and the percentage of the bacilli collected were investigated.

Second-stage phosphate precipitations were shown to increase the concentration of the bacilli for microscopic examination and to provide a means of collecting the bacilli after treatment with acids prior to cultivation.

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## A STUDY OF THE GROWTH OF HUMAN TUBERCLE BACILLI ON A NONPROTEIN SYNTHETIC MEDIUM\*

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IT IS well established at present that human tubercle bacilli grown on relatively poor nutrient media (from the standpoint of composition and of supporting growth from small plantings) can survive for a long time.<sup>1</sup> During this period they show certain morphologic changes in structure (cytomorphosis<sup>2</sup>), which parallel the curves of active growth; then pass into relative quiescence (or senescence) when certain specific materials are formed and liberated. Within the past several decades, the study of the bacilli and their products has been simplified markedly by the development of simple nonprotein synthetic media, such as those of Long and Seibert, Henley and LeDue, and Wong and Weinzirl, to mention only a few. Our own interest originated from the desire for more exact studies of the significance of the tubercle bacillus and its products from the standpoint of specific reactions, among which are included mainly specific immunity and specific intoxication.<sup>3</sup> For this purpose, it became pertinent that a simple inexpensive medium be utilized and that the products be standardized sufficiently to unify the materials used for specific tests, particularly from a quantitative standpoint. After exhaustive preliminary trials with various combinations of essential constituents such as salts, amino acids, glycerol, and glucose, the Wong-Weinzirl medium<sup>4</sup> was chosen because it gave an excellent growth within several months with all the human strains of tubercle bacilli tested, was easily prepared uniformly, was inexpensive, and was a simple nonprotein medium. It proved comparatively nontoxic when injected intravenously into animals, especially after the greater part of the glycerol had been utilized for growth by the bacilli. The aims of these experiments primarily were to choose a suitable medium for our purpose by comparative tests and to study the conditions under which the maximum growth of tubercle bacilli and their liberated products occurred. This required studies of the mass of growth at various intervals, the utilization of some of the important chemical constituents of the medium, the relation of volume and surface of the medium to growth, and finally, the amount of essential products liberated and found in the medium. To a minor extent, some of these factors had been studied previously for short periods. Seibert,<sup>5</sup> using Long's asparagin medium for a maximum interval of twelve weeks, found that the tuberculo-protein content in the medium increased after five to six weeks from 0.1 Gm. per liter to 0.5 Gm. per liter at twelve weeks. In 1937 Wong<sup>6</sup> studied the relation of tuberculo-protein formation to

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growth of the culture in various media. He found that on an inexpensive glycerol-free synthetic medium (containing glucose as a substitute for glycerol) approximately three times as much protein may be obtained as from Long's synthetic medium at the end of six weeks. He concluded that a terminal alkaline pH is an important factor in the rapid production of tuberculin. His tests ran only to ten weeks in these experiments.

#### METHODS

*Culture Bottle.*—In earlier studies it was found advisable to use small neck bottles for long duration incubation of liquid media in order to avoid evaporation of the liquid as far as possible. More recently, with improvement in the grade of bottle glass, it was found advisable to take advantage of a previously described device,<sup>7</sup> the cork inner lined screw cap bottle. On test these showed that the cork would dry sufficiently in the upright position to allow easy access of air, as tested by actual manometric side arm attachments, and yet would maintain absolute sterility for years. Such a bottle with culture is shown in Figs. 2 and 4. These bottles can be obtained in various sizes to give equal surfaces with variable volume content and equal volumes with variable surfaces of liquid.

*Tuberculo-protein* was determined by trichloroacetic acid precipitation (Seibert).

*Glycerol* was determined colorimetrically by the Kolthoff method (modified for our purposes). To 5 c.c. of the solution, 2 c.c. 4 N phosphoric acid and 3 per cent potassium permanganate are added. After ten minutes 1 c.c. of 10 per cent oxalic acid solution is added and agitated. The solution becomes light brown in color in one to two minutes; then 1 c.c. of dilute sulfuric acid and 5 c.c. of Schiff's reagent are added. A red violet color appears and is compared with a standard glycerol solution similarly treated.

*Glucose* was determined by the Folin and Wu method for reducing sugars.

The *bacillary mass* was determined by filtration through a Buchner funnel, washing thoroughly to free from adhering salts and drying to constant weight at 100° C.

#### RELATION OF VOLUME AND SURFACE OF MEDIUM TO GROWTH OF TUBERCLE BACILLI

In 1934 Dorset and Henley<sup>8</sup> stated that "the time required for the cultures to reach full growth is influenced not only by the relation between the volume and surface area of the medium" (a nonprotein asparagin, dextrose, glycerol medium was used) "but also by the amount of medium in the culture flask, even though the same ratio between volume and surface area be maintained. For example, cultures planted on 100 c.c. of medium with a surface area of approximately 60 sq. cm. will reach full growth approximately 2 weeks earlier than cultures planted on 200 c.c. of medium with a surface area of 120 sq. cm." This information was not sufficient to determine the optimum liquid volume to be used, in the liter or any other size bottle, for obtaining the maximum growth of tubercle bacilli at the desired period of about three months. In order to elucidate this more fully, various size and capacity screw cap bottles, from 5



TABLE I  
ANALYSIS OF HUMAN TUBERCLE BACILLI CULTURES ON WONG-WEINZIRL MEDIUM AFTER MAXIMUM GROWTH

AGE OF CULTURE	TOTAL GM. DRIED BACILLI	GM. BACILLI PER 100 C.C. MEDIUM	EVAPORATION IN C.C.	pH	MG. TUBERCULO-PROTEIN PER C.C.	MG. TUBERCULO-PROTEIN PER 100 C.C. ORIG. MEDIUM*	PER CENT GLYCEROL AFTER GROWTH	GM. GLYCEROL USED PER 100 C.C. ORIG. MEDIUM*	PER CENT GLUCOSE AFTER GROWTH	GM. GLUCOSE USED PER 100 C.C. ORIG. MEDIUM*
2 mo.	0.79	1.05	75-40	6.0	0.14†	7.6	1.2	5.3	0.79	0.33
2 mo.	1.03	1.03	100-75	5.2	0.12	8.0	1.4	5.3	0.69	0.48
2 mo.	1.50	1.00	150-125	5.4	0.14	11.6	1.0	5.5	0.79	0.34
2 mo.	2.45	1.23	200-175	6.0	0.28	24.5	1.1	5.4	0.76	0.35
3 mo.	3.97	1.32	300-260	6.6	0.65	56.3				
3 mo.	3.75	1.25	300-260	5.2	0.41	35.5				
3 mo.	5.16	1.28	400-315	6.8	0.61	48.0				
5 mo.	9.05	1.13	800-740	6.8	0.20	18.5				
5 mo.	4.54	0.91	500-400	7.0	0.26	20.8				
5 mo.	11.59	0.97	1,200-1,010	6.1	0.14	11.8	1.0	5.5	0.71	0.43
6 mo.	1.29	0.86	150-115	6.0	0.31	23.8	1.3	5.2	0.50	0.58
6 mo.	4.98	0.99	500-420	6.9	0.34	28.6	1.3	5.3	0.72	0.44
6 mo.	11.98	0.80	1,500-1,125	6.3	0.20	15.0	1.2	5.3	0.86	0.28
7 mo.	1.17	0.78	150-130	6.9	0.54	46.8	1.0	5.6	0.67	0.56
7 mo.	4.10	0.82	500-425	7.0	0.47	39.9	1.0	5.4	0.68	0.41
7 mo.	15.24	1.02	1,500-1,225	7.0	0.31	25.3	1.1	5.6	0.81	0.31
							1.0	5.5	0.78	0.36

\*Calculated.

†Seltz filtrate (30 c.c. filter 3 cm. pad) was used for these tests. The figures may be low because of adsorption of the protein by the filter.



These results indicate that the growth of human tubercle bacilli averages about 1 Gm. of dried bacillary mass per 100 c.c. of medium and that the mass weight decreases gradually in the succeeding months following maximum growth. The tuberculo-protein content of the medium is a factor of the age of the culture. The glycerol consumption of the growing human tubercle bacilli is a constant (about 5 Gm. per 100 c.c.) with relation to the maximum growth. On the other hand, the glucose consumption was smaller in the Wong-Weinzirl medium, about 0.3 to 0.6 Gm. per 100 c.c. being used during the process of attaining maximum growth. Following maximum growth of the human tubercle bacilli on the Wong-Weinzirl medium, a fluid remains which varies in tuberculo-protein content with age and which still contains 1 per cent glycerol and about 0.5 to 0.8 per cent glucose. The pH also ranges from about 6.0 to a final 7.0 after six months' incubation at 37.5° C.



FIG. 2.—The growth of avirulent human tubercle bacilli at seven weeks on buffered salts (5 Gm. ammonium citrate, 2 Gm. sodium carbonate, anhydrous, 6 Gm. monopotassium acid phosphate, 1 Gm. magnesium sulfate, 0.05 Gm. ferric ammonium citrate, 1 liter distilled water) containing different proportions of Wong-Weinzirl medium. Bottles from left to right: full strength Wong-Weinzirl medium, 2 per cent, 1 per cent, 0.5 per cent, and 0.1 per cent. Note growth down to 1 per cent. Similarly, 1 per cent glycerol alone with the salt mixture gives appreciable growth.

#### THE FORMATION OF TUBERCULO-PROTEIN

In order to study in more detail the formation of tuberculo-protein from the bacillary mass and liberation into the liquid medium, the liquid medium was syphoned off aseptically at various periods after growth of the bacilli had begun, and the liquid was replaced by a volume equal to that removed of a buffered neutral salt mixture, essentially the Wong-Weinzirl medium without dextrose, glycerol, and ammonium malate (consisting of 5 Gm. ammonium citrate, 6 Gm. monopotassium acid phosphate, 2 Gm. anhydrous sodium carbonate, 2 Gm. sodium chloride, 1 Gm. magnesium sulfate, 0.05 Gm. ferric ammonium citrate, and 1,000 c.c. distilled water). This neutral salt mixture was used for two reasons: first, that it was almost identical in composition and reaction to the

original nutrient Wong-Weinzirl medium; and second, it would not support the growth of the human tubercle bacilli regardless of the usual amount of planting. However, after two weeks a perceptible additional growth had taken place on the surface of the liquid which, when shaken down, would again be replaced by a small growth after another two weeks. It was found later in a graded control test, as a check upon the foregoing experiment, that as little as 1 per cent of the Wong-Weinzirl medium added to the nonnutrient salt mixture was sufficient to support in small amounts the growth of human tubercle bacilli (see Fig. 2). In further tests it was also found that a small amount of growth occurred when 1 per cent glycerol was added to the nonnutrient salt mixture. The factors of probable growth-stimulating material present in the large mass of tubercle bacilli, as well as a small amount of adsorbed nutrient solution which could not be removed adequately, precluded the use of this method for studying the formation of tuberculo-protein (by decomposition of the bacilli) without the complicating factor of new growth occurring.

TABLE II

pH AND TUBERCULO-PROTEIN LIBERATED IN CULTURES OF VIRULENT HUMAN TUBERCLE BACILLI (pH 7) ON WONG-WEINZIRL MEDIUM

AGE IN WEEKS	TUBERCULO-PROTEIN MG. PER C.C.*	pH	COLOR OF CULTURE LIQUID
4	0.03	6.4	Light yellow
6	0.05	6.5	Light yellow
8	0.18	6.6	Light yellow
10	0.30	6.6	Light yellow
12	0.38	6.4	Golden
14	0.39	6.8	Golden
16	0.46	6.9	Golden
18	0.31	6.6	Golden
18	0.43	7.0	Light gold
20	0.64	7.0	Dark gold
22	0.62	7.0	Light gold
24	0.54	6.5	Dark gold
24	0.58	7.2	Dark gold
26	0.55	7.0	Light brown
28	0.54	7.1	Light brown
30	0.50	7.4	Golden
32	0.73	7.2	Light brown
32	0.68	7.0	Dark gold
34	0.55	7.0	Light brown
36	0.57	7.1	Light brown
38	0.65	7.0	Light brown
40	0.68	6.9	Light brown
42	0.53	6.8	Dark gold
42	0.88	7.0	Light brown
44	0.90	7.0	Dark brown
44	0.74	7.3	Golden
46	0.73	7.0	Dark brown
48	0.95	7.0	Dark brown
48	0.65	7.0	Light brown
50	0.80	7.0	Dark brown
52	0.65	7.0	Dark brown
58	0.77	7.0	Dark brown

\*The tuberculo-protein determinations were made on the centrifugate to avoid adsorption removal by filtration.

We found data in the literature on tuberculo-protein formation only for intervals up to twelve weeks from the time of the planting of the bacilli. In order to obtain more information the tests were extended far enough to deter-

mine completely whether age is the predominating factor in the liberation of tuberculo-protein, and our experiments were carried on for fifty-eight weeks. It has been our practice to plant a number of quart bottles of Wong-Weinzirl medium (containing 500 c.c. of medium) at weekly periods with various strains of virulent and avirulent human tubercle bacilli. These bottles were analyzed after various periods of incubation, starting at four weeks and continuing at two-week intervals thereafter up to fifty-eight weeks. The results of these analyses for tuberculo-protein and the pH are recorded in Table II. The tuberculo-protein curve is plotted in Fig. 3.

An examination of the data recorded in Table II and Fig. 3 reveals that the liberation of tuberculo-protein into the liquid medium may vary within a relatively wide range for individual tests for both virulent and avirulent human tubercle bacilli. As a whole, however, it tends to increase with age to a maximum of about 0.5 to 1.0 mg. tuberculo-protein per cubic centimeter. It is significant that none, or very little, tuberculo-protein is found in the liquid medium during the active stage of growth of the bacillary mass. There seems to be little correlation between pH or color of the liquid and the tuberculo-protein content.

TABLE III

RELATIONSHIP BETWEEN AGE, BACILLARY MASS, AND TUBERCULO-PROTEIN LIBERATED BY CULTURES OF VIRULENT HUMAN TUBERCLE BACILLI ON THE WONG-WEINZIRL MEDIUM

AGE OF CULTURE IN MONTHS*	LOSS BY EVAPORATION† FROM 500 C.C.	WEIGHT IN GM. OF DRIED BACILLI	WEIGHT IN GM. OF TUBERCULO-PROTEIN
1	12‡	2.94	0.01
2	20	4.29	0.06
3	85	5.10	0.16
4	90	4.58	0.19
5	110	4.53	0.17
6	80	3.35	0.39
7	70	3.55	0.17
8	80	3.26	0.37
9	100	3.14	0.24
10	60	3.14	0.33
11	60	2.68	0.36
12	90	2.97	0.32
13	80	3.02	0.27
14	90	3.05	0.35
16	60	2.44	0.36

\*There appears to be no definite correlation between the color and the age of culture or the tuberculo-protein content of the medium.

†All the cultures recorded in this table were grown on 500 c.c. of medium in narrow neck screw cap 1 liter (quart) bottles.

‡All cultures were neutral or slightly alkaline (pH 7 to pH 7.4) except those prior to two months, which were usually about pH 6.5.

In a further experiment to show the correlation between the weight of bacillary mass (as dried weight of bacilli), the age of the culture, and the amount of tuberculo-protein in the liquid media at various ages, these determinations were made on a series of 1 quart (1 liter) culture bottles containing 500 c.c. of Wong-Weinzirl medium. The experiment was carried for over a year. The findings in this test are recorded graphically in Table III.

In general, the results recorded in Table III indicate that the maximum mass of culture from the growth of virulent human tubercle bacilli on the simple Wong-Weinzirl nonprotein medium under the conditions outlined here is

original nutrient Wong-Weinzirl medium; and second, it would not support the growth of the human tubercle bacilli regardless of the usual amount of planting. However, after two weeks a perceptible additional growth had taken place on the surface of the liquid which, when shaken down, would again be replaced by a small growth after another two weeks. It was found later in a graded control test, as a check upon the foregoing experiment, that as little as 1 per cent of the Wong-Weinzirl medium added to the nonnutrient salt mixture was sufficient to support in small amounts the growth of human tubercle bacilli (see Fig. 2). In further tests it was also found that a small amount of growth occurred when 1 per cent glycerol was added to the nonnutrient salt mixture. The factors of probable growth-stimulating material present in the large mass of tubercle bacilli, as well as a small amount of adsorbed nutrient solution which could not be removed adequately, precluded the use of this method for studying the formation of tuberculoprotein (by decomposition of the bacilli) without the complicating factor of new growth occurring.

TABLE II

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\*The tuberculoprotein determinations were made on the centrifugate to avoid adsorption removal by filtration.

We found data in the literature on tuberculoprotein formation only for intervals up to twelve weeks from the time of the planting of the bacilli. In order to obtain more information the tests were extended far enough to deter-

attained at about from two to three months' incubation period. After that time no appreciable growth occurs, and there is a visible shrinkage in bacillary mass (Fig. 4) paralleling the loss in weight of bacillary mass up to the sixteen-month interval. (The same is true for avirulent human tubercle bacilli.) The loss in mass may approach almost 50 per cent of the weight. The liberation of tuberculo-protein into the medium does not begin appreciably until growth has practically ceased, does not account for the total loss in bacillary mass, and is erratic to some extent, but shows an approximate maximum figure in about six to ten months at incubator temperature.

#### SUMMARY AND CONCLUSIONS

1. There is an optimum volume of nutrient medium (Wong-Weinzirl) required for the maximum growth of human tubercle bacilli (three months after planting) for a definite surface area.

2. Analysis of cultures of human tubercle bacilli in bottles of various sizes and surface areas with various volumes of nutrient medium (Wong-Weinzirl) showed that the glycerol consumption was a constant (about 5 Gm. being utilized per 100 c.c. medium) after maximum growth had been obtained. Comparatively smaller amounts of glucose were utilized, and the tuberculo-protein content, though variable, increased with the age of the culture.

3. Growth requirements for the human tubercle bacillus (heavily planted) are not great in a buffered neutral salt mixture (essentially Wong-Weinzirl medium without glycerol, glucose, or ammonium malate). As little as 1 per cent Wong-Weinzirl medium or 1 per cent glycerol added to the salt mixture will support the growth of human tubercle bacilli.

4. The tuberculo-protein present in the liquid medium is not appreciable in 500 c.c. volume of Wong-Weinzirl medium in 1 liter bottles until after maximum growth of human tubercle bacilli has been approached at about two to three months after planting. The liberation of tuberculo-protein into the liquid medium increases to a maximum with the aging of the bacillary mass up to from six to ten months, and probably is a product of autolysis of the bacilli.

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## TECHNIQUE FOR PRODUCING CAROTID LOOPS IN DOGS\*

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THE determination of systolic blood pressures in experimental animals may be made satisfactorily over long periods of time by the use of the carotid loop. Van Leersum<sup>1</sup> described a method employing its use, but its construction was associated with a high morbidity and mortality rate. Child and Glenn<sup>2</sup> later reported a modification of the van Leersum method that gave a higher percentage of successes. The purpose of this paper is to present in detail still another modification of the van Leersum carotid loop in dogs, which, in our experience, has proved satisfactory.

The size and age of the dog are not important. Animals with long necks and animals with considerable loose skin on the ventral surface of the neck are better specimens for the operation as the skin can be pulled together with less tension. The skin of the animals should be smooth and free from disease.

The operative site is prepared by shaving the neck after lathering it well with soap and water. Excess soap is removed with ether and alcohol. It is best to shave the necks of animals just before the operation. If the shaving is done the day before operation, numerous infected papules appear over the skin surface. Operation in the presence of infection usually ends in disaster. Child and Glenn suggest the use of the Schick Dry Shaver.

The dog is next anesthetized with ether and transferred to the operating table. His legs are securely tied to the table. A half roll of toweling paper is placed under the neck to elevate the operative field. The prepared skin is then painted with 7 per cent tincture of iodine, which is allowed to oxidize for thirty seconds before being removed with alcohol. The operative field is draped with four sterile towels.

It is essential that the incisions for the skin flap be made along the course of the carotid artery. It is also important that the skin flap be wide enough so that when the edges are sutured together around the exteriorized carotid artery there will be no necrosis when postoperative edema develops.

The first incision is made just lateral to the midline of the trachea. The incision starts 1 cm. below the thyroid cartilage and extends caudalward and slightly obliquely lateralward to a level of 2 cm. anterior to the sternal notch. The lateral incision is the same length and parallels the medial incision. The two incisions should be about 2 to 2.5 cm. apart, depending upon the size of the animal. The skin flap is now undermined throughout its entire length from the subcutaneous tissue. The superficial external jugular vein is exposed where it

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lies beneath the skin flap. An incision is made just medial to the course of the external jugular vein through the platysma and sternocleidomastoid muscles, splitting the muscles for a distance equal to the length of the skin incisions. The carotid sheath containing the common carotid artery, the internal jugular vein, and the vagus nerve will be found lying just beneath the sternocleidomastoid muscle and lateral to the trachea. The common carotid artery is separated from the rest of the structures of the carotid sheath by careful blunt and sharp dissection throughout a distance of a little greater than the length of the skin flap.

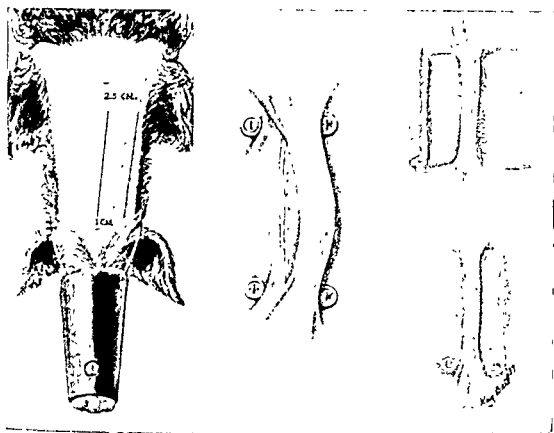


Fig. 1.

The common carotid artery is lifted from its bed and surrounded by the skin flap. The edges of the skin flap are sutured around the artery by a subcuticular No. 1 chromic catgut stitch. This procedure is best done by a large thin curved cutting needle.

The next step in the operation is very important. Two button retention silk sutures are placed through the skin and subcutaneous fascia at each extremity of the skin flap in order to relieve the skin tension at the ends of the new formed loop, permitting union at these two vital points. Separation of the skin edges of the ends of the loop predisposes to infection, thrombosis, and hemorrhage. We have not had a single separation or failure since using these retention sutures. They are removed the eighth or tenth postoperative day.

The muscle incision is now closed with three interrupted equally spaced chromic catgut sutures. The medial and lateral skin incisions are sutured together with a subcuticular No. 1 chromic catgut suture. The suture lines are painted with 2 per cent gentian violet solution.

The type of dressing and the postoperative care of these animals is very important. A strip of gauze is inserted under the loop, and two gauze rolls

are placed parallel to it in such a manner as to prevent its being crushed. Two pieces of gauze bandage are then placed over the loop which is held in position by 10 to 12 layers of gauze wrapped around the neck covering the operative site. Three 1 inch strips of adhesive tape are placed around the neck to hold the dressings. The dressings are changed every other day, and any secretions around the loop are removed with a little soap and warm water. The incisions are again painted with 2 per cent gentian violet solution, and the same type of dressings is applied. These dressings usually can be discontinued after the eighth postoperative day. By the tenth day the postoperative edema has usually subsided so that the loop may be used for blood pressure readings.

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## A STABLE CAPILLARY GLASS ELECTRODE FOR MEASURING THE PH OF LIVING TISSUE\*

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A NUMBER of improvements have been made during the past few years in systems of amplification and of increased stability for glass electrodes of high resistance.<sup>1, 2, 5-7, 10-12</sup> Unfortunately, some of these modifications have resulted in considerable increase in the complexity of the system, and have demanded elaborate shielding, resulting more than often in a very expensive piece of equipment.

In 1936 Skow and Wynd<sup>8</sup> described a nul-point apparatus of considerable simplicity and cheapness, which appeared to be well suited for use with capillary glass electrodes of high resistance of the type to be described presently. With a few minor modifications the amplifying system used here was that described by Skow and Wynd. With this apparatus the major part of the equipment can be enclosed in a grounded metal box, 12 inches by 7 inches by 6 inches. As the system was finally set up, the lead to the glass electrode was practically the only external source of instability. Fairly simple shielding, and a few elementary precautions took care of this difficulty. The lead consisted of shielded metal cable. All measurements were made from a separate table, from which the cable was supported, where necessary, by rubber bands hanging from wooden clamps. It is important that this lead does not, either directly or indirectly, touch the table carrying the amplifying system.

A bakelite support was used for the electrode holder. The buffers used on the animals tested were placed on a small dissecting board with bakelite feet.

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which, in turn, rested on a flat sheet of bakelite. After this part of the apparatus was placed in a wooden box, no further shielding was necessary. The electrodes could thus be manipulated in an animal without the operator being hampered by metal cages or other screening. Undoubtedly, the fact that the work was carried on in a small, partially enclosed, inside room of a concrete building aided in dispensing with elaborate shielding. Even with the grid hanging free, actual physical contact with the wire was necessary to deflect the galvanometer.

In the past one of the interferences with the use of the capillary glass electrode has been the presence of a "deviation film"<sup>4</sup> on the unimmersed portion of the capillary. A large number of substances, such as asphaltum, de Khotinsky cement, ceresin, etc., have been used by various workers to eliminate this source of error, but none of them are of more than temporary value, since fluid soon penetrates beneath the insulating layer.

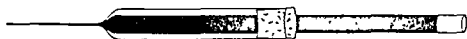


Fig. 1.

In 1935 Varney<sup>9</sup> described a double-shank glass electrode of the bulb type. It occurred to me that a somewhat similar glass shield might be made for the capillary electrode, thereby eliminating completely the "deviation film." After considerable experimentation the laboratory glass blower succeeded in preparing capillary electrodes, similar to the one shown in Fig. 1. Briefly, the procedure was as follows: A spindle was drawn on the outer tube. The inner tube was inserted and fastened firmly at the upper end by cork or de Khotinsky cement. With the latter an air outlet was provided. A closed ring seal was made at the bottom. The spindle was allowed to cool, and then reheated with a fine flame to draw out the capillary.

Using an ordinary vacuum pump, the capillary was filled with one-tenth normal hydrochloric acid, saturated with quinhydrone. Then, by keeping the fluid in the capillary under slight pressure during the process, and using an oxygen flame, capillaries 50 to 60 mm. in length could be sealed with fluid down to the very tip. Longer capillaries were more sensitive and less easily broken, but for the experimental work in hand, namely, determination of the pH of the rat intestine, a capillary 20 to 25 mm. in length proved most suitable. The capillaries were from 40 to 50  $\mu$  outside diameter and from 20 to 30  $\mu$  inside diameter. Before use sealed capillaries were brought to equilibrium by soaking them for twenty-four hours in one-tenth normal hydrochloric acid saturated with quinhydrone; they were kept in this solution whenever they were not being tested.

Satisfactory electrodes, calibrated with standard buffers over the ranges used (pH 5.5 to pH 7.8), gave linear graphs (Fig. 2) and showed a voltage difference of from 40 to 50 mv. for one pH unit. Calibration was made at 37.5° C. With the apparatus already described, both the entire calomel half cell and the glass electrode could be brought to any desired temperature. Gradual changes

in the composition of the glass resulted in a slow alternation of the electrode readings. This usually amounted to 5 or 6 mv. over the course of as many days; consequently, recalibration was advisable every two or three days. With a good electrode and the system of amplification described above, it was possible to make readings accurately and rapidly to a difference of 0.0025 pH units.

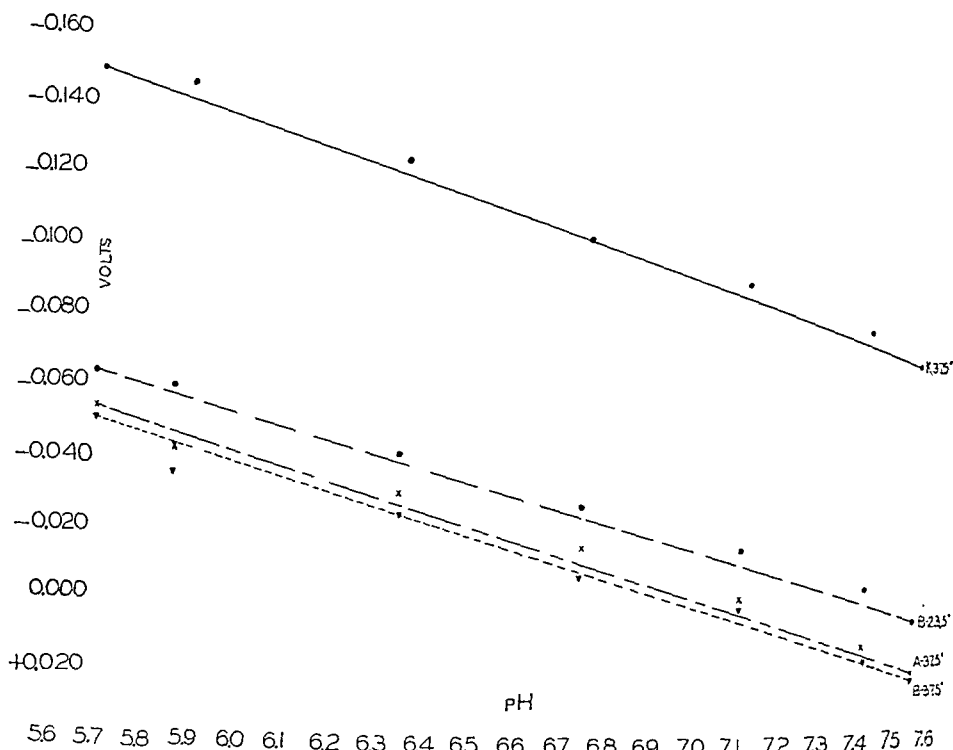


Fig. 2.

Due to the elimination of the deviation film, these electrodes showed a high degree of stability after reaching equilibrium. In one series of readings of a buffer kept at a constant temperature, the electrode showed no demonstrable difference in reading, that is, a variation greater than 1/10 mv. over a period of three hours. Another electrode over a period of six and one-half hours gave the following readings for one buffer at a constant temperature:

TIME	POTENTIOMETER READING—VOLTS
2:23 P.M.	-0.0057
4:27 P.M.	-0.0051
4:35 P.M.	-0.0050
9:00 P.M.	-0.0060

This stability is illustrated also in the reproducibility, using the same series of buffers twice over and in a different order. Such a series is shown in Table I. The maximum deviation was 1.7 mv. or less than 0.05 pH.

An electrode of this type can be used for the determination of the hydrogen-ion concentration of living tissue with a minimum disturbance of the organism. Although the electrode may be inserted into a living cell, the effect of such

TABLE I

BUFFER pH	TIME INTERVAL BETWEEN READINGS	FIRST READING VOLTS	SECOND READING VOLTS	DIFFERENCE IN MV.
5.57	1 hour	-0.0555	-0.0551	0.4
6.25	1 hour 5 minutes	-0.0257	-0.0274	1.7
6.48	2 hours	-0.0163	-0.0148	1.5
6.99	—11 minutes	+0.0045	+0.0056	1.1
7.31	1 hour 45 minutes	+0.0175	+0.0165	1.0
7.46	1 hour 18 minutes	+0.0239	+0.0238	0.1

penetration, as Chambers and Pollack<sup>3</sup> have shown by means of indicators, is to alter the intracellular pH. The electrode is particularly useful for insertion into natural cavities, such as the lumen of the digestive tract, where it may be used to determine the pH of adjacent areas or of limited regions of the tract. It can also be used to determine the pH of different parts of small animals where the bulkier bulb-type electrode would not be suitable.

## SUMMARY

A stable capillary glass electrode is described in which the "deviation film" is completely and permanently eliminated. No elaborate shielding is necessary, and comparatively simple amplification systems can be employed. The electrode can be used to determine the hydrogen-ion concentration of very small areas of living tissue.

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## DETERMINATION OF TOTAL AND FREE CHOLESTEROL IN BLOOD SERUM\*

IRVING ALLAN KAYE, A.B., M.Sc., BROOKLYN, N. Y.

IN 1936 Drekter, Sobel, and Natelson<sup>1</sup> published a paper in which they mentioned a method for the determination of free cholesterol wherein free cholesterol is precipitated with digitonin in an 80 per cent alcoholic medium, and is determined colorimetrically by the procedure of Schoenheimer and Sperry.<sup>2</sup> This method has been in use in the chemical laboratories of this hospital for over three years. During this time several modifications have been introduced which simplify the method further and increase its accuracy. The purpose of this communication is to describe this method in greater detail, together with the suggested improvements, and to present, in addition, a rapid colorimetric method for the estimation of total cholesterol.

Total cholesterol is usually determined by the same procedure as is used for free cholesterol after saponification of cholesterol esters. This procedure, though theoretically sound, is usually very lengthy. To obviate this difficulty the colorimetric reaction of Liebermann and Burchard was applied directly to the lipid extract of cholesterol and cholesterol esters, in spite of the fact that Yasuda<sup>3</sup> proved that the latter give a proportionately deeper color than free cholesterol. However, the percentage of free cholesterol in normal individuals was found to be remarkably constant and of the same order of magnitude as that reported by experimenters who employ the Schoenheimer and Sperry<sup>2</sup> technique using saponification. This reason, and above all, its simplicity and rapidity, justify the presentation of this method.

Cholesterol and its esters are extracted at room temperature by a large volume of alcohol-ether solution. After centrifugation the supernatant liquid is decanted and evaporated to dryness. Total cholesterol is then determined by adding a solution of acetic anhydride and chloroform, followed by concentrated sulfuric acid, directly to this residue. The deep green color which develops is compared with a suitable standard in a microcolorimeter. The simplicity and rapidity of this method are due mainly to the elimination of the time-consuming transfers usually necessary when other procedures are followed. In addition, a stable solution of cholesterol in acetic anhydride has been substituted for the usual standard in chloroform.

The free cholesterol in the alcohol-ether residue is determined by precipitation as the digitonide in 80 per cent alcohol. After the precipitate is washed free of esters with ether, it is dissolved in glacial acetic acid and determined colorimetrically, using as standard a solution of cholesterol and digitonin

\*From the Pediatric Research Laboratory, the Jewish Hospital of Brooklyn.  
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in acetic acid and acetic anhydride. This solution eliminates the necessity of comparing the unknown with known amounts of cholesterol carried through the entire procedure.

In this procedure somewhat larger amounts of serum are used than in the Schoenheimer and Sperry method because no photoelectric colorimeter or photometer was available. However, since few clinical laboratories are equipped with these expensive instruments and since small amounts of serum are used withal, these procedures may be followed wherever a rapid accurate procedure is desired for the determination of free and total cholesterol.

The method may be used to advantage wherever a complete lipid fractionation on serum is desired. The extraction method of Schoenheimer and Sperry with alcohol-acetone reagent cannot be used here because of the relative insolubility of some lipids in this medium, especially phosphatides, whereas extraction with Bloor's alcohol-ether mixture is an accepted procedure. The weighed lipid residue may be dissolved in cold benzene, and free and total cholesterol may be determined on aliquots of this solution after evaporation of the solvent at 60° C.

#### METHOD

##### *Reagents.*—1. Total cholesterol standard.

One hundred milligrams of pure dry cholesterol are dissolved in 250 c.c. of acetic anhydride (1 c.c. = 0.4 mg. of cholesterol).

By comparison from time to time with equal amounts of pure cholesterol, this solution has been found to be stable for at least eight months.

##### 2. Digitonin solution in glacial acetic acid.

Dissolve 175.6 mg. of digitonin in a little glacial acetic acid. Warm to 60° C. to hasten solution, cool, then dilute to 100 c.c. with the same solvent. This solution is stable for at least a year.

##### 3. Free cholesterol standard.

To 25 c.c. of the total cholesterol standard add 25 c.c. of the digitonin solution in glacial acetic acid and 25 c.c. of acetic anhydride. Mix well in a glass-stoppered bottle (3 c.c. = 0.4 mg. of cholesterol). This solution has been found to be stable for at least one year.

##### 4. Digitonin solution, 0.5 per cent.

Dissolve 500 mg. of digitonin in 100 c.c. of 50 per cent alcohol (55 c.c. of 95 per cent alcohol and 45 c.c. of distilled water) at 60° C. This solution is stable for at least four months.

##### 5. Alcohol-ether solution.

Add 750 c.c. of 95 per cent alcohol to 250 c.c. of ether. Keep in a cool place in a glass-stoppered bottle.

##### 6. Alcohol, 95 per cent.

7. Concentrated sulfuric acid. Baker's C. P. product was used and kept in a small dropping bottle.

8. Glacial acetic acid, ether, acetic anhydride, and chloroform. Baker's C. P. products were used.

9. Acetic anhydride-chloroform solution. This reagent must be prepared immediately before use or stored in a refrigerator for a period not greater than two weeks. For each 10 c.c. of acetic anhydride add 20 c.c. of chloroform.

*Total Cholesterol.*—Twelve cubic centimeters of alcohol-ether solution are added in a rapid stream to 0.2 c.c. of serum in a 15 c.c. test tube. A finely divided protein precipitate is formed which is easily packed on centrifugation. After centrifuging for three minutes at 2,000 r.p.m., the supernatant liquid is decanted into a 25 c.c. Erlenmeyer flask and evaporated just to dryness on a water bath.\* When it is thoroughly dry and cool, 3 c.c. of the acetic-anhydride-chloroform solution are added, followed by 2 drops of concentrated sulfuric acid. The contents of the tube are thoroughly mixed, stoppered, and set aside for ten to fifteen minutes for color development. The standard is prepared at the same time by adding 2 c.c. of chloroform to 1 c.c. of the cholesterol standard in acetic anhydride. Two drops of sulfuric acid are added, mixed, and the color is permitted to develop out of direct light for ten to fifteen minutes. The colors are compared in a microcolorimeter.

*Free Cholesterol.*—Twenty-five cubic centimeters, of alcohol-ether solution are added in a rapid stream to 0.5 c.c. of serum in a 30 c.c. test tube. After centrifugation at 2,000 r.p.m. for three minutes, the supernatant liquid is decanted into a 50 c.c. beaker and evaporated to dryness on a water bath. The lipids are transferred quantitatively to a 15 c.c. centrifuge tube with three 2 c.c. portions of ether. The ether is evaporated to dryness by placing the test tube in a container of warm water. Two cubic centimeters of 95 per cent alcohol are added, and the test tube is stoppered and placed in an oven or water bath at 60° C. for about thirty minutes. One cubic centimeter of digitonin solution is then added, and the contents of the tube is thoroughly mixed. The test tube should be left at room temperature for at least three hours before being centrifuged for ten minutes at 2,000 r.p.m. The supernatant liquid is aspirated, and the precipitate is suspended in 3 c.c. of ether. After centrifuging for five minutes, the supernatant liquid may be decanted without fear of disturbing the precipitate. The precipitate is washed once more with ether, dried at 60° C., and dissolved in 1 c.c. of glacial acetic acid at the same temperature. After cooling to room temperature, 2 c.c. of acetic anhydride and 4 drops of concentrated sulfuric acid are added. The solution is mixed well and set aside for twenty-seven minutes out of direct light for color development. Color comparisons are made in a microcolorimeter with a standard prepared at the same time by adding 4 drops of concentrated sulfuric acid to 3 c.c. of the digitonin-cholesterol solution.

### Calculation—

Total cholesterol:

$$\text{Mg. per 100 c.c.} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{mg. of cholesterol in the standard} \times 500$$

Free cholesterol:

$$\text{Mg. per 100 c.c.} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{mg. of cholesterol in the standard} \times 200$$

\*Overheating of the dry residue must be avoided. For this reason, the last traces of solvent are sometimes evaporated in a water bath or oven at 60° C.

## RESULTS

The results of a large number of determinations carried out under routine analytical conditions, without too much emphasis on technique, are presented in Table I. An alcoholic solution of cholesterol was added to the lipid extract of blood serum, and the solvent was evaporated. The procedure from this stage on was the same as is described under the procedure for free cholesterol. In addition, a pure solution of cholesterol in 95 per cent alcohol was treated with digitonin, and the amount of cholesterol digitonide found by the same procedure. The results in Table I were accumulated during the past two years in our laboratories where it is a routine practice to check on the validity of methods and reagents.

TABLE I  
RECOVERY OF FREE CHOLESTEROL BY THE DIGITONIN METHOD

NATURE OF RECOVERY	NUMBER OF DETERMINATIONS	AMOUNT OF CHOLESTEROL ADDED	MEAN AMOUNT RECOVERED	AVERAGE DEVIATION FROM MEAN	STANDARD DEVIATION OF MEAN
Cholesterol added to lipid extract	42	0.400 mg.	0.3906 mg.	± 0.0131	0.01383
Cholesterol added to lipid extract	20	0.200 mg.	0.2004 mg.	± 0.0094	0.0127
Pure solution	152	0.400 mg.	0.3891 mg.	± 0.0107	0.0131

In Table II are presented results carried out under research conditions, with more attention to details. While these results are superior to the former, it is apparent that the method is fairly accurate even when carried out under the conditions found in the average clinical laboratory.

TABLE II  
REPRESENTATIVE RECOVERIES OF FREE CHOLESTEROL BY THE DIGITONIN METHOD

NATURE OF RECOVERY	AMOUNT OF CHOLESTEROL ADDED	AMOUNT RECOVERED
Cholesterol added to lipid residue	0.400 mg.	0.396 mg.
Cholesterol added to lipid residue	0.400 mg.	0.390 mg.
Cholesterol added to lipid residue	0.400 mg.	0.410 mg.
Cholesterol added to lipid residue	0.400 mg.	0.396 mg.
Cholesterol added to lipid residue	0.200 mg.	0.204 mg.
Cholesterol added to lipid residue	0.200 mg.	0.199 mg.
Cholesterol added to lipid residue	0.200 mg.	0.195 mg.
Cholesterol added to lipid residue	0.200 mg.	0.199 mg.
Pure alcoholic solutions of cholesterol	0.400 mg.	0.400 mg.
Pure alcoholic solutions of cholesterol	0.400 mg.	0.400 mg.
Pure alcoholic solutions of cholesterol	0.400 mg.	0.398 mg.
Pure alcoholic solutions of cholesterol	0.400 mg.	0.394 mg.

In Table III are listed the results of 25 analyses on normal individuals. It is to be noted that while the absolute amounts of total and free cholesterol by this procedure are variable, the percentage of free cholesterol in the total cholesterol is fairly constant. Sperry<sup>4</sup> in his studies observed that his percentages were slightly higher, varying from 24.3 per cent to 30.1 per cent. This is probably due to the fact that our total cholesterol values, where no saponification was employed, are higher than his.

TABLE III  
VALUES ON NORMAL INDIVIDUALS  
(Expressed in mg. per 100 c.c. of serum)

TOTAL CHOLESTEROL	FREE CHOLESTEROL	% FREE CHOLESTEROL	TOTAL CHOLESTEROL	FREE CHOLESTEROL	% FREE CHOLESTEROL
215	55	26	278	79	28
237	58	25	257	71	28
273	64	23	185	48	26
188	52	28	154	41	27
174	43	25	162	41	25
183	44	24	174	48	28
246	56	23	240	57	24
213	53	24	202	54	26
209	46	22	203	49	24
222	60	27	218	60	28
259	61	23.5	195	55	28
231	64	28	Normal range 154-273	Normal range 41-71	Normal range 22-28
226	59	26			
238	55	23			

Table IV contains the results of the method of extraction presented in this paper compared with four other widely used methods. Bloor,<sup>5</sup> Kirk, Page, and Van Slyke,<sup>6</sup> Boyd,<sup>7</sup> and Dreker, Bernhard, and Leopold<sup>8</sup> were the experimenters whose methods were chosen. In the Bloor technique twenty volumes of alcohol-ether solution were refluxed for about five minutes with one volume of serum. The refluxing was continued for one hour in the case of Kirk and co-workers; extraction and no refluxing at all were permitted in the Boyd extraction. The protein precipitate was filtered and washed free of lipids with hot alcohol-ether solution in the case of the first two extractions, and with the cold reagent in the latter method. The combined filtrates were evaporated to dryness, and the residue was dissolved in an amount of benzene equivalent to the amount of serum used in the initial extraction (3 c.c.). One-half cubic centimeter aliquots were pipetted into centrifuge tubes for the digitonin procedure, and 0.2 c.c. aliquots were used for the determination of total cholesterol. The benzene was evaporated in an oven at 60° C., and the determinations were continued as described in the regular procedure.

TABLE IV  
FREE AND TOTAL CHOLESTEROL BY VARIOUS METHODS OF EXTRACTION  
(Pooled serum was used)

AUTHOR'S			KIRK, PAGE, AND VAN SLYKE			BOYD			DREKTER AND BERNHARD			BLOOR		
CHOLESTEROL			CHOLESTEROL			CHOLESTEROL			CHOLESTEROL			CHOLESTEROL		
TOTAL	FREE		TOTAL	FREE		TOTAL	FREE		TOTAL	FREE	%	TOTAL	FREE	%
mg. %	mg. %	%	mg. %	mg. %	%	mg. %	mg. %	%	mg. %	mg. %	%	mg. %	mg. %	%
237	58	25	242	55	23	228	59	26	224	52	23			
			224	50	22	215	55	26						
350	66	19	288	62	21.5	253	67	26.5	300	63	21	288	61	21
302	77	25.5	308	64	21	310	69	22	296	62	21	305	64	21
273	64	23	257	64	25	256	66	26	253	64	25	262	62	24
			254	58	23	259	61	23.5						

In the dry extraction of Dreker, Bernhard, and Leopold, 3 c.c. of serum were added to fat-free filter paper, which was then dried at room temperature.



The cholesterol was extracted by the vapors and condensate of a 3 per cent pyridine solution in 95 per cent alcohol. The apparatus used was a simplified Soxhlet extraction apparatus. A large test tube, with a constriction that supported the filter paper curled into a tightly-packed ball, was loosely stoppered with a mushroom water condenser. About 8 c.c. of the solvent were added, the mushroom condenser was set in place, and the tube was placed in a boiling water bath for one hour. At the end of this time the water in the condenser was turned off, and the solvent was permitted to evaporate slowly. The last drop of solvent was removed under vacuum. The residue was dissolved in benzene, and the determination continued as described for the other extraction methods.

The results of Table IV and of other unpublished work indicate that occasionally my method of extraction gives higher values for total cholesterol, but the ratio of free cholesterol to total cholesterol is about the same. These lower values may be due to the fact that the other extraction methods are not as complete as the one described. The somewhat low free cholesterol percentages observed may be due to the action of cholesterol esterase on the pooled serum which had been standing for some time.

#### SUMMARY

Simple and accurate methods for the determination of free cholesterol and total cholesterol in blood serum have been described.

I am indebted to Dr. Albert E. Sobel for his invaluable assistance in the presentation of this investigation.

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## URINE BROMIDES\*

### A NEW QUALITATIVE TEST

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THE fact that bromides may cause intoxications with psychotic symptoms has been known for many years, but not until the methods of determination of blood bromide by Ulrich in 1910, Walters in 1912, Hauptman in 1925, and Wuth in 1927, did clinicians realize the importance of this type of mental aberration. Following these investigators, numerous other workers have attacked the problem of bromide intoxications, and while most complete clinical studies have been carried out, it is still the consensus of opinion<sup>1-3</sup> that a definite diagnosis of bromide intoxication can be made only by the identification of bromide in the urine or blood. Wile<sup>4</sup> further pointed out that the urine in some cases may be free of bromide when the blood bromide is high; hence the blood should always be examined. Such cases are rare, however, and for practical clinical purposes, it may usually be assumed that the possibility of a bromide intoxication may be eliminated by demonstrating the absence of bromide in the urine. This is fortunate, for while Hauptman and Wuth both described methods for blood bromide determination, the average clinician seldom has the facilities even if he has the time to make these studies. For this reason, a simple method of qualitatively determining bromide in urine is of considerable practical use, provided one always remembers that certain cases of kidney disease, fluid retention, or chloride deficiencies may show an elevated blood bromide, although but small amounts of bromide are excreted in the urine.

One of the earliest and most widely used methods of determining urine bromide was described in 1927 by Wuth<sup>5</sup> as follows:

"To 25 c.c. of urine about 1 Gm. of animal charcoal is added, mixed well, allowed to stand a few minutes and then filtered. To exactly 5 c.c. of filtrate measured into a test tube, 1 c.c. of 20 per cent trichloroacetic acid and 1 c.c. of 0.5 per cent gold chloride solution is added; a brown shade indicates the presence of bromides."

This method is accurate and fairly easy, but the reagents used are seldom found in the average laboratory and, moreover, are very expensive.

In the same volume of the *Journal of the American Medical Association*, is also found an article by Belote,<sup>6</sup> wherein he describes a simple qualitative test for urinary bromides.

"Small strips of filter paper are soaked in a saturated solution of fluorescein in 60 per cent acetic acid. These are then allowed to dry and

\*From the Department of Psychiatry of the New York State Psychiatric Institute and Hospital, New York.

Received for publication, September 26, 1939.

may be kept indefinitely as indicators of the test. The suspected body fluid is placed in a test tube. To this are added a few crystals of potassium permanganate. After agitation, a few drops of concentrated sulphuric acid are added and the fluorescein paper is held, after moistening with two per cent acetic acid, at the mouth of the test tube. The presence of even minute amounts of bromine is at once indicated by a rapid change in color from the original yellow to a bright pink on the paper."

This method, while easy and rapid, is somewhat too sensitive, as it will show bromides in a concentration of 1:40,000, and is, therefore, not practical for clinical determinations in suspected cases of bromide intoxication.

Perhaps the simplest method has been described by Todd and Sanford<sup>7</sup> as follows:

"Bromides can be detected by acidifying about 10 c.c. of the urine with dilute sulphuric acid, adding a few drops of fuming nitric acid and a few c.c. of chloroform and shaking. In the presence of bromine, the chloroform, which settles to the bottom, assumes a yellow color."

This test is especially useful because of the universal availability of the reagents required, and because it is so easy to perform. Unfortunately, however, it is not accurate except with rather large concentrations of bromide. As it is described it is of little practical use for clinical purposes.

The need for a simple, easily performed test, however, was met by my modification of this method as follows:

To 10 c.c. of urine add 0.1 c.c. dilute sulfuric acid, 2 c.c. of fuming nitric acid, and 5 c.c. of chloroform. Shake the mixture thoroughly. Thus far this is essentially the foregoing method. At this point, if there is a relatively high bromide concentration—over 250 to 300 mg. per hundred cubic centimeters—the chloroform settling to the bottom will give a yellow to orange red color, because the bromide is oxidized by the acids to form free bromine which is then taken up by the chloroform. If iodides are present, they impart to the chloroform a purple to violet red hue. It was found, however, that if after thoroughly agitating the mixture described, 1 c.c. of chlorox (5.25 per cent sodium hypochlorite) was added, amounts of bromine as small as 75 mg. per hundred cubic centimeters could accurately be detected. Such minute amounts give the chloroform a greenish-yellow tinge. Chlorox alone when mixed with the aforementioned acids and chloroform in plain water, or urine in the absence of bromide, colors the chloroform a faint green; this color can be easily distinguished from the green yellow color produced by bromide concentrations of 50 mg. to 75 mg. per hundred cubic centimeters or higher. In order to determine the coloring formed by the chlorox alone, it is probably best to set up a trial run of the various reagents in bromide-free urine or water, so that the observer will become familiar with the shades of color produced. Larger amounts of bromide in the urine produce a change in colors from green yellow to yellow to yellow orange to orange to orange red to red.

The rationale of this procedure is easy to understand if one recalls that free chlorine, as is present in the nascent state in chlorox, owing to its higher

position in the atomic table, will replace bromide, in turn liberating free bromine. The chlorox, therefore, promotes a more complete liberation of all the bromine, which in turn is taken up by the chloroform. A much greater amount of the available bromine is set free, and the test is accurate for concentrations as low as 50 mg. to 75 mg. bromide per hundred cubic centimeters of fluid. This method is somewhat similar to the one described in the early German literature by Neuberg,<sup>8</sup> who employed either nitrous acid or chlorine water but never combined the two reagents. He warned against this method since it was difficult to detect the bromine unless large amounts were present. However, this objection has been overcome in the present modification of the test, and the test is now sufficiently sensitive for clinical qualitative estimates. Quantitative estimates can even be made fairly accurately by the evaluation of the various color changes. Quantitative urine bromide determinations are, however, of almost no clinical value because of the marked individual variability in bromide elimination, depending upon the chloride balance of the body fluids, kidney elimination, water balance, and other changeable factors. Qualitative findings are sufficient, and if 50 to 100 mg. of bromide per hundred cubic centimeters of urine are found (green yellow to yellow color), accurate quantitative blood estimates are imperative.

The presence of iodides is indicated by a purple or red violet color of the chloroform following the agitation of the mixture previous to the addition of the chlorox. Because chlorox bleaches out this color, it is important to shake thoroughly the mixture of urine, sulfuric acid, nitric acid, and chloroform, and then permit it to stand for a few seconds to observe any coloring of the chloroform before the chlorox is added.

This test is especially simple, requiring but a minute for a complete determination. All the reagents, dilute sulfuric acid, concentrated nitric acid, chloroform, and chlorox are usually available in even the smallest laboratory. Furthermore, a test tube and a measuring graduate or pipette are the only pieces of apparatus required.

#### SUMMARY

An attempt to review some of the more usual clinical methods of the qualitative demonstration of bromide in the urine has been made, and a new modification of the Todd and Sanford method has been described.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**BONE MARROW BIOPSIES, Clinico-Hematologic Evaluation of, Morrison, M., and Samwick, A. A.** *Am. J. M. Sc.* 198: 758, 1939.

A study was made of 275 patients with sternal bone marrow aspiration.

Bone marrow ratios were utilized in evaluating: (a) leucogenesis; (b) erythrogenesis; (c) erythrocytic maturative defect; (d) infiltration of bone marrow by lymphocytic, plasma, reticulum, and malignant cells.

Bone marrow aspirations proved valuable in the diagnosis of gastric dysfunction and gastrointestinal, liver, and renal disease.

This study revealed a close relationship between the erythrocytic maturative defect and color index.

Criteria for the diagnosis of blood dyscrasias, including pernicious anemia, Hodgkin's disease, leucemia, agranulocytosis, infectious mononucleosis, polycythemia vera, infections, and malignancies are suggested.

**TUBERCULIN TEST, Its Value and Limitations, Long, E. R.** *Am. Rev. Tuberc.* 40: 607, 1939.

Recently reported experience has prompted much discussion of the tuberculin test, and made desirable a revaluation of the test with indication of its limitations.

The conditions of human observation make the determination of the specificity and adequacy of the tuberculin test more difficult in man than in animals. The experience of B.C.G. vaccination, however, has shown that in the overwhelming majority of human beings, artificial infection brings about tuberculin sensitivity, just as it does in laboratory guinea pigs. Sanatorium and hospital experience also indicates that a still more overwhelming majority of patients obviously suffering from pulmonary tuberculosis react positively to tuberculin.

Occasionally persons are encountered, however, in whom evidence of tuberculosis exists, but the tuberculin reaction is negative. At present there are only rough indications of how frequently this occurs. The few surveys made in which both tuberculin testing and x-ray examination have been carried out suggest that in ordinary practice the intracutaneous test with reliable samples of old tuberculin or purified protein derivative tuberculin will detect 90 to 95 per cent of cases of tuberculosis.

The loss of 5 to 10 per cent of persons is attributable in large part to the variable conditions of tuberculin allergy. In any large survey, particularly of children, a few persons are likely to be found in whom infection has occurred, but the reaction has not yet become positive. Subsequent films in such cases may show lesions. Subsequent retest with tuberculin yields a positive result, but often the retest is not made.

A more important reason for the failure of the tuberculin test to detect all tuberculosis is the waning and disappearance of allergy that occur with healing. In this country of dropping tuberculosis morbidity and increasing isolation of patients with open tuberculosis, this is probably a phenomenon of increasing tendency. It is apparently true that unless allergy is stimulated from time to time by reinfection, in cases of progressive healing of an existing infection, the intensity of response will diminish.

**SULFANILAMIDE, Anemia Induced in Rats by, Machella, T. E., and Higgins, G. M.** *Am. J. M. Sc.* 198: 804, 1939.

Anemia can be induced in rats by the administration of sulfanilamide.

The degree of anemia depends on the dose used and the duration of administration.

The anemia is accompanied by evidences of stimulation of bone marrow, that is, reticulocytosis, macrocytosis, anisocytosis, and leucocytosis.

No change in level of platelets has been detected.

Complete recovery from the anemia is possible following cessation of administration of the drug.

A second course of sulfanilamide will reproduce the anemia.

The drug is capable of producing toxic symptoms in rats, the symptoms depending on the dose employed. Symptoms include cyanosis, loss of weight, thirst, gastric distention, drowsiness, irritability, and convulsions.

The anemia, when induced by moderately toxic doses, is associated with a significant hepatomegaly and splenomegaly.

**TUBERCULOSIS, Sulfanilamide in,** Corper, H. J., Cohn, M. L., and Bower, C. *Am. Rev. Tuberc.* 40: 452, 1939.

Large administrations of sulfanilamide over long periods of time, parenterally as well as enterally, begun before or coincident with infection of guinea pigs with virulent human tubercle bacilli, have an apparent, though not real, effect upon the organic tuberculous involvement of these animals which is most strikingly evident in the spleen. These findings explain the earlier, cautiously presented studies on enteral treatment by Rich and Folis, and subsequently by Greey and colleagues, which could not be verified by parenteral sulfanilamide treatment studies.

The apparent effect noted upon the tuberculosis in the guinea pig is explained as an organic toxic effect of the sulfanilamide, since it can also be noted when heat-killed and avirulent human tubercle bacilli are given intravenously in large amounts to animals which are treated as compared with untreated controls.

Even in large doses, sulfanilamide has no appreciable effect upon the spread of tuberculosis in guinea pigs infected by various routes and in various ways with virulent human tubercle bacilli; such effect as is seen can be entirely explained on the basis of the toxic effect of the sulfanilamide and not upon a retardation of the tuberculosis.

In elaboration of the view expressed by Shecket and Price (1939) that "the use of sulfanilamide in conditions in which its value is not established should be reserved for cases under institutional direction" and that the drug should not be used for long duration administration, the authors add that misinterpretation of the effect in chronic diseases especially should be avoided by carefully controlled experimental studies considering the organic toxic action of the drug before application to human treatment.

**BURN SHOCK, Study of,** Trusler, H. M., Egbert, H. L., and Williams, H. S. *J. A. M. A.* 113: 2207, 1939.

The tannic acid theory of the treatment of burns is fallacious. There is no local application that can be expected to save life after a large burn.

The first cause of death due to the extensive burn is shock. There are two phases in the burn shock reaction.

(a) The traumatic phase: Circulatory shock due to vascular stasis and the loss of blood plasma, which escapes into the tissue through capillaries injured by heat. Very large burns may be rapidly fatal by this mechanism.

(b) The inflammatory phase: A complex syndrome in which the loss of fluid is accompanied by the other morbid processes of diffuse thermal inflammation. This accounts for the toxic manifestations.

The blood chemical changes of the shock state depend largely on the manner in which fluids are administered. The indiscriminate forcing of simple fluids into a burned patient is futile and may lead to profound disturbances in the physical-chemical relations of the blood.

The persistent forcing of water by mouth under these conditions may cause a fatal water intoxication.

The successful treatment of burn shock calls for a complex therapeutic regimen. Blood transfusions should be given early and repeated frequently throughout the self-limited period of the reaction. Other fluids should be given in moderate quantities. Large amounts of dextrose are indicated.

**HEMOCONCENTRATION, Occurrence and Clinical Significance of, Moon, V. H. Ann. Int. Med. 13: 451, 1939.**

Reported observations on hemoconcentration indicate that this phenomenon occurs rather frequently in grave conditions of disease quite diverse in origin. Undoubtedly this survey is far from complete, but the number of instances found is sufficiently large and diversified to justify a summary of the author's observations and interpretations.

It appears that hemoconcentration is regularly associated with a type of circulatory deficiency in which loss of plasma volume is the essential feature.

The loss of fluid may result either from endothelial damage which allows for leakage of plasma into the tissue spaces, or from dehydration incident to vomiting, diarrhea, and perspiration. This loss may be compensated in part by absorption of fluid from the tissues and in part by constriction of the vascular walls, especially the heart, arteries, and spleen and, to a lesser degree, by constriction of the veins and capillaries.

So long as the mechanism of compensation is effective, no marked deficiency is evident. When compensation becomes inadequate, the blood pressure declines progressively, anoxia develops, and the syndrome of shock is manifested.

So long as the tissue endothelium is able to perform its part in the maintenance of fluid balance, there is dehydration of the tissues but not of the blood. In advanced stages of shock the vascular system is able neither to absorb nor to retain fluid. It appears that the critical point in this mechanism is the physiologic state of the vascular endothelium.

This type of circulatory deficiency may develop whenever and however the capillaries in an extensive visceral area are rendered atonic.

A rising curve of concentration is as ominous as a falling curve of arterial pressure. But the former occurs early and indicates the developmental stage of circulatory deficiency while the latter indicates the failure of compensation and the imminence of death.

Circulatory failure in its incipient stage may be recognized by the presence of hemoconcentration. This feature is of inestimable practical value, for treatment must be applied early to be effective.

**BLOOD, Preservation of Stored, With Sulfanilamide, Novak, M. J. A. M. A. 113: 2227, 1939.**

Contamination of stored blood with bacteria has not been considered seriously enough in the past.

Many unexplained transfusion reactions may be due to pyrogens or other bacterial by-products in contaminated blood.

Complete bacteriostasis of the usual bacterial contaminants in stored blood is made possible by the addition of 20 mg. per hundred cubic centimeters of sulfanilamide. Such blood will not only not support bacterial growth for ten to fifteen days but may actually become sterile in that time.

Sulfanilamide may prove valuable as a preservative in other biologic substances.

**BLOOD BANK, Plasma Prothrombin Content of, Lord, J. W., and Pastore, J. B. J. A. M. A. 113: 2231, 1939.**

On the basis of the method of Warner, Brinkhous, and Smith, bank blood is an adequate source of plasma prothrombin for about nine days. At longer intervals of storage the plasma prothrombin declines gradually, reaching the level of 61 per cent of normal by the end of the third week of storage.

Carefully controlled refrigeration of bank blood is an important factor in the preservation of plasma prothrombin.

**BOTULISM, Demonstration of Toxin in Blood and Tissues, Schneider, H. J., and Fisk, E. J. A. M. A. 113: 2299, 1939.**

Five outbreaks of botulism, with three fatal and three nonfatal cases, occurred within a period of ten months in Los Angeles County.

Circulating toxin was demonstrated in the blood of one patient by animal injection and its specificity (type A) determined by toxin-antitoxin experiments. Circulating toxin could not be demonstrated in three nonfatal cases.

Toxin was demonstrated in saline liver extracts in all the fatal cases.

The demonstration of circulating or tissue toxin as herein described should aid in the diagnosis of botulism. The authors believe that such attempts are indicated in all suspected cases of botulism.

The procedures used follow:

In investigating the possibility of active toxin in the organs of the deceased person twenty hours after necropsy and twenty-six hours after death, 50 Gm. of liver was triturated and extracted with 50 c.c. of physiologic solution of sodium chloride; 1 c.c. of this extract was injected intraperitoneally into each of two guinea pigs, one protected with 0.5 c.c. of mixed antitoxin (A and B). After thirty-six hours the unprotected guinea pig developed experimental botulism and died forty-two hours after injection. The protected guinea pig appeared normal at this time but died suddenly a few hours later without the development of any signs of botulism. Graded doses of this extract, when injected into mice several days later, gave conflicting results, the evidence being suggestive but not conclusive.

Blood collected at the time of admission to the hospital prior to antitoxin administration was tested for the presence of botulinus toxin in the following manner: Ten mice, 5 of which had been previously injected (fifteen minutes) with botulinus antitoxin (A and B) were injected intraperitoneally with 0.5 c.c. of the citrated blood. At the end of thirty-six hours one unprotected mouse died and the remaining 4 showed symptoms of experimental botulism, viz., shallow breathing, muscular spasms, especially of the abdominal muscles, and later paralysis of the hind limbs and prostration. Three mice died in forty-eight hours, and one sixty hours after injection. All the protected mice survived without symptoms.

**BLOOD UREA, Experimental Variation of, Drevermann, E. B. M. J. Australia II: 74, 1939.**

The blood urea at any particular time is dependent on the previous diet, the fluids taken, and the condition of work or rest under which the blood is taken.

The rise in blood urea following a gastrointestinal hemorrhage results in part from the formation of urea from blood proteins which are digested in the intestine. The diminished excretion of urea from compensatory retention of fluid also contributes to this effect.

**HEPATITIS, Toxic, Kirshbaum, J. D., and Popper, H. Arch. Int. Med. 65: 463, 1940.**

Fifteen cases of acute hepatitis are described. The fulminating fatal form appears to be an intermediary stage between catarrhal jaundice and acute yellow atrophy of the liver. The liver in each case was enlarged and characterized morphologically chiefly by damage to the epithelial cells and serous hepatitis, with dissociation of the liver cells. The clinical diagnosis was usually considered as primary hepatitis.

The dissociation of the cords of liver cells may be the result of severe serous hepatitis consequent to damage of the blood capillaries of the liver.

Icterus in parenchymatous jaundice is due to the combination of localized necrosis of the liver cells, or destruction of the cords, and general impairment of the function of the liver cells (serous hepatitis).

The enlargement of the liver in the authors' cases and in cases of catarrhal jaundice is due to toxic edema.



**LIVER, Colloidal Gold Reaction of Blood Serum in Diseases of the, Gray, S. J. Arch. Int. Med. 65: 524, 1940.**

The colloidal gold reaction of the blood serum was studied in a series of 96 cases of hepatic disease, grouped as follows: 46 cases of cirrhosis, 14 cases of acute parenchymatous disease, 25 cases of neoplastic involvement, and 11 cases of miscellaneous hepatic disease. The diagnoses were confirmed by autopsy, biopsy, or laparotomy in 34 cases; 11 in the first group, 2 in the second, 13 in the third, and 8 in the fourth. The reaction was positive in each of 46 cases of cirrhosis, in 13 of 14 cases of acute parenchymatous involvement, in 19 of 25 cases of neoplasm, and in each of 11 cases of miscellaneous hepatic disease. In the entire series, the reaction was positive in 89 of the 96 cases, or in 92.7 per cent.

In several instances the colloidal gold reaction detected liver disease later confirmed by operation or autopsy, which had not been detected by the usual clinical and chemical tests.

The colloidal gold reaction was negative with sera from 20 normal adults and in 73 of 75 persons with various extrahepatic diseases; normal livers were found in 22 of these persons at autopsy, biopsy, or laparotomy.

Positive reactions were obtained in 8 of 20 cases of syphilis. In one of the 8 there was a history of hepatitis, but in the others there was no demonstrable hepatic disease. The possibility of latent damage to the liver following arsenical therapy and the question of false positive reactions in syphilis are discussed. It is pointed out that the colloidal gold reaction of the spinal fluid and that of the blood serum may be the same or may differ greatly, depending on the effects of disease processes on the permeability of the blood-brain barrier.

The Takata-Ara reaction was positive in only 34 of 58 cases of hepatic disease (58.6 per cent); it was thus considerably less sensitive than the colloidal gold reaction.

The total plasma cholesterol was normal in 38 of the 77 cases of hepatic disease studied.

However, decreased values for plasma cholesterol esters were frequently associated with extensive parenchymal damage and were present in 47 of 71 cases of hepatic disease (66.2 per cent). The decrease paralleled the severity of the hepatic injury and was of prognostic significance; 6 patients whose cholesterol esters were too low to determine died within two months.

Changes in the cholesterol-cholesterol ester ratio paralleled the shifts in the plasma proteins in all types of liver disease except the acute parenchymatous diseases, in which lowered cholesterol esters were more frequent than altered plasma protein values.

Variations in the plasma proteins were frequent in hepatic disease; globulin was increased in 43 cases, and albumin was decreased in 45 of the 75 cases studied. The increase in globulin was usually greater than the decrease in albumin, so that the total proteins were normal in 60 of the 75 cases.

The most marked albumin-globulin shifts occurred in cirrhosis of the liver and were associated with advanced hepatic destruction; the least marked variations were found in the acute parenchymatous hepatic diseases. The changes in concentration of the plasma proteins were related more closely to the severity and duration of the hepatic disease than to the state of nutrition or to the loss of protein associated with ascites.

The colloidal gold reaction does not depend primarily on a quantitative increase of globulin or on a low or inverted albumin-globulin ratio. Positive reactions were obtained in 29 cases of hepatic disease with normal plasma globulin values and in 21 cases with normal albumin-globulin ratios. The mechanism of the reaction is unknown. It is suggested that it may depend on a qualitative rather than on a quantitative variation in the plasma globulin, and that the euglobulin may be an important factor in the reaction.

## REVIEWS

Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

### Physiological Basis of Medical Practice\*

IN THEIR preface the authors remark that, although physiology is a science in its own right and the laboratory worker who pursues his researches quite detached from medical problems need offer no apology for his academic outlook, the teacher of physiology should, nevertheless, emphasize those aspects of the subject which will throw light on disorders of function. Their textbook, therefore, becomes a volume on applied physiology rather than pure physiology. Obviously, this is a very praiseworthy approach as long as the fundamental knowledge of pure or normal physiology is adequately covered at the same time.

As one looks over the first few chapters on blood and lymph, with their discussion of blood chemistry, erythropoietic diseases, hemocytometry, edema, transfusion, blood grouping, hemolysis, the chemistry of hemoglobin, and the classification of the anemias, one wonders whether this will turn out to be a volume on physiology, biochemistry, clinical pathology, or diagnosis. As soon as one reaches the sections on circulation and respiration, one realizes how adequately the field of physiology is covered and welcomes the continued interjection of remarks on the application of physiology to the study or treatment of specific disturbances thereof.

A volume which has gone through five printings and now appears as a new edition in its sixth printing since 1937 must already have established its own popularity. While this is undoubtedly due in large measure to its popularity as an undergraduate text, the fact that through its pages one may find information which can be applied in the study and intelligent treatment of disease, makes it equally valuable as a reference volume for the practicing physician.

In this second edition a new section, The Physiology of the Special Senses, has been added.

### Lee on the Levee†

IN THIS era of science and acute technocracy, only too often is the machine glorified at the expense of the man. It was not that Stradivari had better tools; the superiority was in the man. All who are professionally interested in the expert laboratory work universally done in medicine today with the superb equipment available, should pause to note that Dr. William Beaumont did his classic experiments on the stomach of the wounded Alexis St. Martin "with no more equipment than a thermometer, a few open-mouth vials, and a sand bath."

A graphic dramatization of the Alexis St. Martin shooting accident at Mackinac Island in 1822, and a most sympathetic characterization of Dr. Beaumont is included in the new historical novel *Lee on the Levee*. The story covers the friendship that existed between Dr. Beaumont and Robert E. Lee, resulting from the residence of their two families together in the Governor William Clark mansion on the St. Louis waterfront in 1834, while Lieutenant Lee was doing his invaluable engineering work on the Mississippi. The story is in effect an opportunity to visit with Lee and Beaumont as actual living men going about their daily tasks and talking about their past achievements and future hopes and ambitions.

\*The Physiological Basis of Medical Physiology. By Charles Herbert Best, M.A. and Norman Burke Taylor, M.D., F.R.S. M.R.C.S. (England), L.R.C.P. (London), Professor of Physiology, University of Toronto, Canada. ed. 2, 1872 pages, 497 illustrations, \$10.00. Williams & Wilkins Co., Baltimore, Md., 1935.

†Lee on the Levee. By Ralph Cannon. Cloth, 188 pages, \$2.50. The Saravan House, New York, N. Y.

The novel is authentically based on a packet of unpublished letters from the Lees to the Beaumonts over a period of fifteen years following their year of residence under the same roof. These letters were recently discovered by Dr. Arno B. Luckhardt, of the University of Chicago, who obtained them from the Beaumont heirs for a museum display.

### Demonstration of Physical Signs in Clinical Surgery\*

THE importance of such a book as this is succinctly stated in inference in the following quotation by Lockwood: "If it is a question of doubt in diagnosis, you may often observe that one man solves the doubt when the others could not, and the way in which one man happened to solve it is this: he applied to the diagnosis of the case some method of examination which the others had not applied."

While the many procedures and devices now applicable to the study and diagnosis of disease have much to commend them in the last analysis, they should never be allowed to take the place of a thorough examination of the patient. This is the text and purpose of Dr. Bailey's book—to emphasize this necessity and to demonstrate by a clearly written text and numerous well-chosen and excellently reproduced illustrations how to demonstrate the physical signs of disease. That he has achieved his purpose is evidenced by the fact that the book is now in its seventh edition.

This book should be owned, read, and reread by every physician. It is well worth many times its cost.

### Experimental Pharmacology and Materia Medica†

A COMPREHENSIVE students' laboratory manual in experimental pharmacology, most abundantly illustrated and with fifty-five exceptionally good color plates. The color plates illustrating the various plants from which drugs are derived are the kind one would like to frame and hang on the wall.

The completeness of the volume is illustrated by the presence of chapters on shop work and laboratory photography. The section on Materia Medica and prescription writing will be of use to graduate physicians. It includes such subjects as posology, incompatibilities, Latin pronunciation, Latin inscriptions, prescription writing, solubilities of common substances, Latin-English and English-Latin vocabularies, and a list of commercial houses from whom various supplies and equipment may be procured.

### Standard Methods‡

IMMEDIATELY upon its appearance the first edition of this book became a standard reference text par excellence for the laboratory and the laboratory worker. Indispensable for the public health laboratory, it is also of great value and utility to the smaller laboratory.

The present edition embodies some changes in technique and new methods and, as before, may be regarded as comprehensive and authoritative. This book can be highly recommended without qualification as a *sine qua non* for the laboratory.

\**Demonstration of Physical Signs in Clinical Surgery.* By Hamilton Bailey, F.R.C.S. (Eng.), Surgeon, Royal Northern Hospital, London; Surgeon and Urologist, Essex County Council; Surgeon, Italian Hospital; Consulting Surgeon, Clifton Hospital and the County Hospital, Chatham; External Examiner in Surgery, University of Bristol. Cloth, ed. 7, 310 pages, 377 illustrations, many in color, \$6.50. Williams & Wilkins Co., Baltimore, Md.

and *Materia Medica*. By Dennis E. Jackson, Ph.D., M.D., Professor of Pharmacology, Materia Medica, and Therapeutics in School of Medicine; Formerly Associate Professor of Pharmacology, Washington University Medical School, St. Louis. Cloth, ed. 2, 906 pages, with 892 illustrations, including 55 color plates. The C. V. Mosby Company, St. Louis, Mo., 1939.

‡*Standard Methods of the Division of Laboratories and Research of the New York State Department of Health.* By Augustus B. Wadsworth, M.D. Cloth, ed. 2, 681 pages, 20 figures, 39 plates, \$7.50. Williams & Wilkins Co., Baltimore, Md.

### Whitla's Dictionary of Treatment\*

SIR WILLIAM WHITLA, renowned as a physician and teacher throughout the scientific world as well as in his native Britain, practiced in Belfast between the years 1877 and 1920. He died in 1933.

Of the three medical books that he wrote, the *Dictionary of Treatment* is outstanding, and throughout seven previous editions it has maintained individual recognition among the most modern and authoritative references for the general practitioner.

The eighth edition, following the author's death, was written, like its predecessors, by members of the Belfast School. Acknowledgment is given for the aid of Dr. H. Gardiner-Hill and Mr. B. W. Williams, physician and surgeon, respectively, to St. Thomas's Hospital, London.

The eighth edition contains over 1,200 pages of text. It treats surgical and medical conditions from the point of view of the general practitioner. The subject matter is conveniently arranged throughout in alphabetical order, and each topic is introduced with a subjective and objective symptom and sign description. General and specific treatment is given, with opinions from the better recognized schools of thought. Specific medication and prescriptions are abundant.

This is indeed an excellent book for brief, comprehensive, and satisfactory reference.

### Argyria†

SILVER has been in use in medicine since the eighth century and argyria is a condition long known; it is also one concerning the mechanism of which there is great uncertainty and of which there has been hitherto to this reviewer's knowledge no systematic study or review.

In this book all that is known of argyria has been comprehensively reviewed. Diagnosis, treatment, prophylaxis are all discussed, as well as argyria as an industrial hazard. An extensive bibliography and list of proprietary silver compounds, together with author and general indices, is appended.

In view of the increasing use of therapeutic silver compounds with the inevitable possibility that argyria may increase as a result, this is a valuable, interesting, and instructive contribution to the subject.

### Disorders of the Blood‡

THE literally enormous literature concerned with hematology is prima facie evidence of the fact that there is no condition which may not be reflected by some change in the blood picture, and emphasizes the importance of blood studies in clinical medicine.

Doctors Whitby and Britton present in this book a comprehensive digest of the pertinent literature. That they have done so satisfactorily is shown by the fact that their book is now in its third edition.

The present edition has been thoroughly revised and increased by new pages and new illustrations.

As before, this is a thoroughly practical text, well deserving a place in the physician's reference library.

\*Whitla's Dictionary of Treatment including Medical and Surgical Therapeutics. By R. S. Allison, M.D., M.R.C.P. (London); and C. A. Calvert, M.B., B.Ch., F.R.C.S.I. Cloth, ed. 8, 1,285 pages, \$9.00. William Wood & Co., Baltimore, Md.

†Argyria. The Pharmacology of Silver. By William R. Hill, M.D., Instructor in Dermatology and Syphilology, University of Pennsylvania; and Donald M. Pillsbury, M.D., Associate Professor of Dermatology and Syphilology, University of Pennsylvania. Cloth, 172 pages, \$2.50. Williams & Wilkins Co., Baltimore, Md.

‡Disorders of the Blood, Diagnosis, Pathology, Treatment and Technique. By L. H. Whitby, M.D., F.R.C.P., D.P.H.; and C. J. Britton, M.D., D.P.H., Bland-Sutton Institute of Pathology, London. Washable fabric, ed. 3, 603 pages, 61 text figures, 12 plates, 3 in color, \$7.50. P. Blakiston's Son & Co., Philadelphia, Pa.

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## *CLINICAL AND EXPERIMENTAL*

### THE EFFECTS OF CIGARETTE SMOKING ON METABOLIC RATE, HEART RATE, OXYGEN PULSE, AND BREATHING RATE

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OUR investigation was originally undertaken as a study of the immediate effects of smoking on metabolic rate and heart rate. Thirty-nine persons were used, 18 men and 21 women. They were grouped as (1) heavy smokers, (2) light smokers, and (3) abstainers. In this series no attempt was made to insure basal metabolic conditions, although no determinations were made directly after meals.

A second series of observations was carried out to determine the effects over a longer period of time. Twenty persons were studied, all in a basal metabolic state. After a preliminary determination of basal metabolic rate, heart rate, breathing rate, and oxygen pulse, the person was asked to smoke a cigarette, expending no more effort than was necessary. A second determination of metabolic rate was made immediately following this, and three more determinations at fifteen-minute intervals thereafter. In all experiments, disturbances, such as bright light, noises, temperature fluctuations, etc., were avoided. Subjective changes reported by the person were noted, as well as the manner of smoking, i.e., whether or not smoke was inhaled.

The apparatus used was a late model McKesson Metabolor. Face masks were used rather than the rubber bit and nose clamp in order to afford the person greater comfort and to facilitate normal breathing.

#### RESULTS

The data obtained in these experiments are shown in the accompanying tables and figures. The persons used in determining immediate effects of smoking (Table I, A) represent an average group of individuals, including heavy

smokers, light smokers, and abstainers. Determinations of metabolic rate were not made under basal (postabsorptive) conditions. The 20 persons used in determining long time effects of smoking were all habitual smokers. Each was in a basal condition and none had smoked since the previous day.

TABLE I

## EFFECT OF SMOKING ONE CIGARETTE

*A*, Immediate effects. *B*, Effects over a 45-minute period. Metabolic rate (calories per square meter per hour); heart rate (beats per minute); oxygen pulse (c.c. oxygen per minute per heart beats per minute); breathing rate (inhalations per minute).

	METABOLIC RATE	HEART RATE	OXYGEN PULSE	BREATHING RATE
<i>A: 39 Persons (Not Basal) Immediate Effects of Smoking</i>				
Previous to smoking	35.1	67.6	3.8	12.2
Immediately after smoking	38.3	71.8	3.7	12.1
Percentage change	8.8	6.2	-2.9	-0.7
<i>B: 20 Persons (All Basal) All Habitual Smokers</i>				
Previous to smoking	31.8	61.6	3.4	12.2
Immediately after smoking	36.3	71.9	3.3	11.3
Percentage change	14.3	16.7	-2.1	-7.4
15 minutes after smoking	34.7	62.8	3.5	11.6
Percentage change	9.3	1.9	5.7	-4.4
30 minutes after smoking	34.7	60.4	3.6	11.6
Percentage change	9.2	-1.9	8.4	-4.6
45 minutes after smoking	35.7	60.0	3.7	12.0
Percentage change	12.4	-2.6	11.0	-1.1

Table I and Fig. 1 show only the average effects and give no indication of the extremes. The greatest increase in metabolic rate in this series occurred in a woman who is a confirmed smoker and inhales the smoke deeply. Her metabolic rate rose 40.7 per cent immediately after smoking. It may be added that in one individual in a state of nervous tension and emotional instability, a rise of 84 per cent was observed; this person was excluded from the data as abnormal. Five individuals showed a decrease in metabolic rate. One individual in the second series (basal persons) showed a decrease, the rate of metabolism falling 8.5 per cent immediately after smoking, continuing to fall fifteen minutes later, after which it returned to nearly normal.

TABLE II

## IMMEDIATE EFFECTS OF SMOKING IN 39 PERSONS

Percentage of total cases showing increase, decrease, and no change in metabolic rate, heart rate, oxygen pulse, and breathing rate.

	METABOLIC RATE	HEART RATE	OXYGEN PULSE	BREATHING RATE
Percentage of persons showing increase	82.0	71.8	55.3*	41.0
Percentage of persons showing decrease	12.8	25.6	44.7	55.9
Percentage of persons showing no change	5.1	2.5	0.0	5.1

\*A greater percentage shows an increase in oxygen pulse, yet Fig. 1 shows a decrease after smoking. This is due to the more profound drop in some persons rather than the rise in others.

By the distribution graph (Fig. 2) it can be seen that in the greatest number of cases an increase in metabolic rate of from 5 to 10 per cent occurred. The average increase was 8.9 per cent. In three cases the maximal metabolic rate

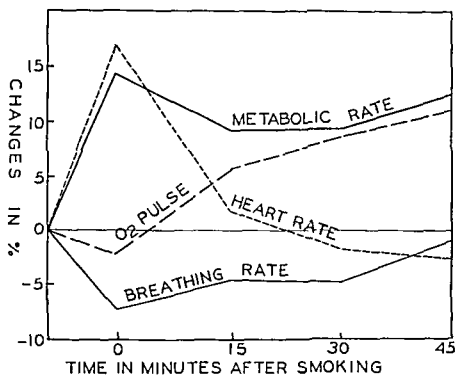


Fig. 1.—Per cent changes in metabolic rate, heart rate, oxygen pulse, and breathing rate over a forty-five-minute period after smoking. Average for 20 persons in basal metabolic state.

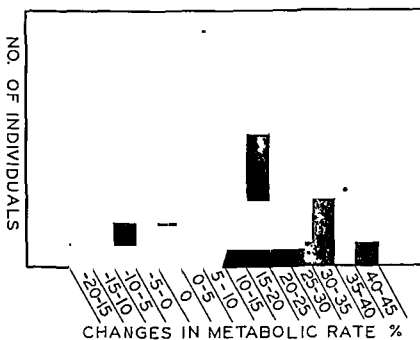


Fig. 2.—Distribution of changes in metabolic rate immediately following smoking in 39 persons. Arrow indicates the average

was reached fifteen minutes after smoking, in three cases at thirty minutes, and in four cases at forty-five minutes. The remaining ten cases showed the maximal metabolism immediately after smoking. Most of the persons showed a second slight rise at about forty-five minutes. In Table II are indicated the percentages of total cases that showed an increase, a decrease, or no change in metabolic rate, heart rate, oxygen pulse, and breathing rate, immediately following smoking. Typically there occurred simultaneously an increase in metabolic rate, an increase in heart rate, a decrease in breathing rate, and a temporary decrease in oxygen pulse, followed by a rise back to and above the normal level.

A comparison of effects of smoking on men and women was made. The average increase in metabolic rate for 18 men was 7.7 per cent; the average

increase for 21 women was 9.9 per cent. The average increase in heart rate for men was 5.9 per cent, while that for women was 6.4 per cent.

In general, there was a tendency for those who inhaled the most smoke to show the greatest physiologic changes. Profound changes also occurred among abstainers and among those who smoked only occasionally, doubtless due to the lack of acquired tolerance. Habitual smokers who inhaled little or no smoke tend to show only moderate effects.

#### DISCUSSION

It is obvious that many factors contribute to the physiologic changes due to smoking, including the individual variation in method of smoking and tolerance for nicotine, the state of health and emotional stability of the subject, the psychic factors involved in making the determinations, the form of tobacco used, and the presence of substances other than nicotine in tobacco.

In this series of experiments psychic factors were reduced to a minimum, though such factors might well be considered important in individuals smoking for the first time, or in those individuals who rarely smoke. In the second group of experiments in which tests were made over a forty-five-minute period, all persons were accustomed to cigarette smoking. No information was given the subjects as to the type of effect to be expected. It will be noted from Table I that persons in a basal state showed more profound changes than did the others. This is in accord with the observations of most smokers that smoking produces greater subjective changes before meals than after meals. It has been found also (Haggard and Greenberg, 1934) that changes in blood sugar and respiratory quotient are greatest in a fasting condition.

The oxygen pulse almost invariably undergoes a diminution immediately after smoking, followed by a rise to a level considerably higher than normal, indicating an increase in oxygen used per stroke of the heart. The initial fall may be attributed to the great increase in heart rate and the resulting decrease in stroke volume occurring before the force of the beat had improved proportionally.

In persons in a basal condition, the effects of smoking one cigarette persist for at least forty-five minutes. The trend of the curves (Fig. 1) indicates that these effects must continue over a still longer period; how long is not shown by the present experiments.

Effects of cigarette smoking are due almost solely to absorbed nicotine, although other volatile substances, such as pyridine and collidine, are present in tobacco smoke, together with some hydrocyanic acid and carbon monoxide (Sollmann). Bodnár, Nagy, and Dickmann (1935) report that 6 per cent of the nicotine is absorbed by "noninhalers," 1.7 per cent of which is excreted in the urine, and that 93 per cent of the nicotine is absorbed by "inhalers," none of which is excreted in the urine. Thus a tolerance is developed which might well account for the great effects sometimes apparent in nonhabituated smokers. It has been demonstrated by many that nicotine in sufficiently large doses stimulates adrenalin secretion and hyperglycemia. Dill, Edwards, and Forbes (1934) found that in 90 per cent of the 60 observations on smoking in ten subjects, the blood sugar level varied within only 5 per cent of the resting



level without significant trend up or down. They found also that blood lactic acid was not varied by smoking. Oxygen consumption was found to increase, in some subjects from 5 to 10 per cent, this being in accord with our results. McCormick (1935) believes the rise in blood sugar is a direct response of the adrenals to the presence of a toxin. He offers the evidence that resistance to nicotine poisoning is greatly increased by previous administration of adrenalin.

Recently Short and Johnson (1939) have shown in a study of five subjects that the changes in pulse rate, blood pressure, and skin temperature due to smoking cigarettes continuously could be duplicated by subcutaneous injection of adrenalin solution (1 c.c. of 1:1,000). They concluded that nicotine effects can be explained by an increased output of adrenalin. Whether the effects of smoking are due only, if at all, to liberation of adrenalin or to other pharmacologic reactions of nicotine will have to be settled when our knowledge is more complete.

#### CONCLUSIONS

1. Cigarette smoking caused an increase in metabolic rate in 82 per cent of 39 subjects. In 13 per cent a decrease occurred. In 5 per cent no immediate effects were observed. The average effect on metabolic rate was an increase of 8.9 per cent.
2. The maximum effect on basal metabolism of smoking one cigarette was reached immediately in some cases, and was delayed as long as forty-five minutes in others.
3. The first rise in metabolic rate was typically followed by a second increase, reaching its summit about forty-five minutes later.
4. Smoking caused an increase in heart rate in 72 per cent of the persons, a decrease in 26 per cent, and no change in 2.5 per cent. After fifteen minutes the heart rate became slower than normal.
5. The rate of breathing decreased immediately after smoking, returning to normal in about forty-five minutes.
6. Smoking caused an immediate reduction in the oxygen pulse value, followed by an increase for at least forty-five minutes.
7. Greatest physiologic effects of smoking were shown by confirmed smokers who inhaled the smoke, and by persons who were unaccustomed to smoking.
8. In this study, women showed more marked changes than men.
9. More marked changes occurred in persons in a basal metabolic condition than in persons in a nonbasal metabolic condition.

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# A COMPARISON OF THE EFFECTS OF LARGE DOSES OF CALCIUM GLUCONATE-IDONATE, CALCIUM GLUCONATE, AND CALCIUM CHLORIDE\*

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THE wide use of intravenous calcium therapy makes the pharmacologic action of a readily soluble calcium salt a matter of some interest. Calcium gluconate-idonate† is a very soluble salt. Hence a study of its action, comparing it with calcium chloride and with calcium gluconate, has been made.

## PROCEDURE AND RESULTS

*Chronic Studies in Rats.*—The drugs in solution or suspension were administered by stomach tube to rats six days weekly throughout the experiment. The animals were weighed at least once a week. Certain tissues from animals dying during the experiment, as well as those surviving for its duration, were examined histologically. Calcium gluconate-idonate, calcium chloride, and calcium gluconate were all given in amounts equivalent to 0.4 Gm. calcium per kilogram of body weight daily, the first two in solution and the third in a heavy suspension. The gluconate-idonate and the chloride were also tested at levels equivalent to 1 and 2 Gm. calcium per kilogram per day. Ten animals comprised each group except that receiving 1 Gm. calcium per kilogram as gluconate-idonate; it contained 20 rats.

All the animals receiving calcium at the 2 Gm. per kilogram level died after a single dose; hence they are not included in Fig. 1, where the average weight of each group is plotted against the survival time in days. A cross bar indicates the death of an animal. Administration of 1 Gm. of calcium per kilogram of body weight as calcium chloride produced death after one or two doses, but as calcium gluconate-idonate 4 animals survived the entire period. In a dose of 0.4 Gm. calcium per kilogram, of the rats receiving calcium chloride 5 of the original 10 survived the entire period, while all those receiving calcium gluconate-idonate or calcium gluconate of the same calcium content survived. Two of the gluconate animals died (at 56 and 63 days, respectively), but the entire gluconate-idonate group was still alive.

*Pathology.*—The heart, kidney, and liver tissue from animals given calcium gluconate-idonate equivalent to 1 Gm. of calcium per kilogram per

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The calcium gluconate-idonate used in this study was supplied by Charles Pfizer & Co. Inc., who have contributed generously to the support of this research.

†Calcium gluconate-idonate is soluble in water to the extent of 411 Gm. per liter of solution at 25° C. It is possible to prepare a temporarily supersaturated solution of about 15 per cent at room temperature. The calcium content of the salt is approximately 9.3 per cent. The acid is the sugar acid derived from the hexose idose and is isomeric with gluconic acid.

day for seventy days were examined histologically. These tissues were essentially normal in appearance, showing no abnormalities which could be ascribed to the effect of the drug. Likewise, neither calcium gluconate for seventy days nor calcium chloride for sixty-five days each, given in amounts containing 0.4 Gm. of calcium per kilogram per day, produced any apparent change in these tissues.

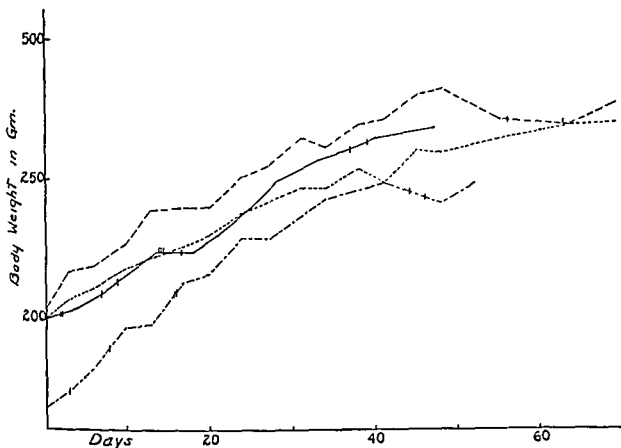


Fig. 1.—Average growth curves of rats receiving indicated doses of calcium salts daily. Cross bars indicate deaths of animals.

- Calcium chloride = 0.4 Gm. Ca per kg.
- ..... Calcium gluconate = 0.4 Gm. Ca per kg.
- · - · - Calcium gluconate-idonate = 0.4 Gm. Ca per kg.
- Calcium gluconate-idonate = 1.0 Gm. Ca per kg.

**Acute Studies.**—Eighteen adult dogs were used. The animals received morphine sulfate subcutaneously, 10 mg. per kg. body weight, thirty minutes before injection of the calcium salt was begun. The calcium solution\* (0.205 M) was injected through a cannula into the femoral vein at varying rates. A blood sample was withdrawn from the femoral vein just prior to cannulation; a second was obtained at death by direct cardiac puncture. The concentration of calcium in the serum was determined by the method of Kramer and Tisdall.<sup>3</sup> Electrocardiograms† from Lead II were taken at frequent intervals during the injection.

The protocols from all experiments are summarized in Table I. The results of Hoff, Smith, and Winkler,<sup>1,2</sup> who used the same procedure with calcium chloride, are included for comparison.

\*To prepare the calcium gluconate solution it was necessary to heat the mixture on the steam bath to bring about solution. Dextrose 5.0 per cent, citric acid 0.37 per cent, and lactic acid 1.0 per cent were then added to stabilize the supersaturated solution which was filtered and diluted to volume after recooling to room temperature.

Calcium gluconate-idonate in 20 per cent solution (0.507 M) was also injected intravenously into each of 2 dogs in quantities of 3 c.c. per kg. at rates of 1.76 and 2.11 c.c. per kg. per minute without subsequent ill effect.

†Dr. H. E. Hoff and Dr. P. K. Smith kindly assisted in obtaining the electrocardiograms.

TABLE I

DOG	CALCIUM CHLORIDE					CALCIUM GLUCONATE- IDONATE					CALCIUM GLUCONATE				
	Kg.	C.C.	C.C./Kg.	C.C./Kg. MIN.	Mg. % CA	Kg.	C.C.	C.C./Kg.	C.C./Kg. MIN.	Mg. % CA	Kg.	C.C.	C.C./Kg.	C.C./Kg. MIN.	Mg. % CA
1	12.2	99	8.1	0.5	51.4	12.5	193	15.4	0.81	111	12.5	856	68.7	1.01	162 <sup>A</sup>
2	9.1	280	30.8	0.7	45.6	9.0	92	10.2	1.12	100	10.8	63	5.8	3.89	182
3	10.5	66	6.3	0.8	53.0	5.8	166	28.6	1.22	231	10.7	110	10.3	1.72	100
4	6.8	149	21.9	0.8	106.4	6.8	296	43.5	1.34	290	10.2	89	8.7	1.29	88
5	12.6	89	7.1	1.0	56.8	6.2	37	6.0	5.97	170	9.0	368	40.9	1.14	173
6	7.5	113	15.1	1.1	70.8	6.9	84	12.2	0.94	76	18.1	368	20.3	0.96	83
7	7.5	142	18.9	1.1	60.0	7.0	214	30.6	1.22	127	12.5	108	8.6	0.86	98
8	8.8	209	23.8	2.6	52.4	10.7	610	57.0	1.66	242 <sup>A</sup>	10.1	203	20.1	1.23	152
9	11.6	86	7.4	7.4	95.4	11.6	524	45.2	1.10	168 <sup>A</sup>					
10	7.0	155	22.1	1.1	126.0	8.0	120	15.0	5.45	110					
11	17.5	230	13.1	1.1	138.4										
12	6.4	142	22.2	1.6	114.0										
13	9.6	43	4.5	2.6	86.0										
	Mean		15.5		81.2			26.4		162.5			17.8		134.5
	S.D.		8.4		31.8			17.4		71.0			12.32		45.9
	PEm		1.57		5.9			3.7		16.0			3.14		10.7

<sup>A</sup>—died in cardiac arrest.

Changes in the electrocardiogram followed the sequence reported by the above-mentioned authors for calcium chloride. An initial phase of inhibition, both of rate and of auriculoventricular conduction, was followed by a period of excitation, during which ectopic rhythms appeared, and which often culminated in ventricular fibrillation. In experiments in which ventricular fibrillation did not appear during the rapid phase, a second period of inhibition occurred, and the animal died of cardiac arrest. In experiments in which calcium gluconate-idonate or gluconate were employed, certain variations were detected in their effect on the heart. First, corresponding with the greater amounts of calcium required to kill, the duration of the various phases was increased. Second, the degree of inhibition during the first stage was increased, so that arrest of as much as eight to ten seconds was observed. Thus, the tendency of the ventricles to fibrillate persisted even after the second slowing phase began, so that ventricular fibrillation frequently terminated a burst of tachycardia after an arrest of fifteen seconds or more. Because of this extension of the period during which ventricular fibrillation might be expected, 8 of 10 animals given calcium gluconate-idonate, and 7 of 8 given calcium gluconate died of fibrillation, compared with 6 of 13 given calcium chloride.

Appreciably larger quantities of calcium as gluconate-idonate or as gluconate are required to produce death than are needed when calcium is given as the chloride. This is reflected in the terminal serum calcium values, the means of those for both organic salts being significantly higher than that with the chloride. The gluconate-idonate and the gluconate do not appear to differ appreciably in their effects. It is possible that the difference between the chloride and the sugar acid salts can be explained in part by the different amounts of calcium ions they yield.

## SUMMARY

Calcium chloride, whether given orally to rats or intravenously to dogs, is definitely more toxic than calcium gluconate-idonate or calcium gluconate.

Calcium gluconate-idonate and calcium gluconate differ very little in their toxicity for dogs and rats. Over a long period of time (seventy days) calcium gluconate proved lethal to 2 of 10 rats, and calcium gluconate-idonate to none of 10.

Intravenous injections of 0.205 M solutions continued to the point of cardiac arrest (usually with ventricular fibrillation) revealed no differences between the effects of gluconate and calcium gluconate-idonate.

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FATAL RENAL INSUFFICIENCY FOLLOWING THE ADMINISTRATION OF SULFAPYRIDINE\*

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THE object of this paper is to describe briefly a case of fatal renal insufficiency accompanied by hematuria and oliguria following the clinical use of sulfapyridine.

## CASE REPORT

*Clinical History.* L. P., an 80-year-old white spinster, was admitted to University Hospitals on April 29, 1939, with a history of an upper respiratory infection of eight days' duration and a severe productive cough of three days' duration. Her past history was essentially negative except for pneumonia three years previously.

*Physical Examination.* Her temperature was 102.9° F., her pulse 80, her respirations 28, and her blood pressure 165 systolic and 85 diastolic. She was a well-developed and well-nourished woman, looking much younger than her stated age. Her throat was red and a slight mucopurulent discharge was present in the pharynx. There was impairment of the percussion note at the bases of both lungs posteriorly, together with numerous coarse and fine crepitant râles. A systolic murmur was noted at the apex. The rest of the physical examination was negative.

*Laboratory Findings.* The urine on admission was entirely negative. The leucocyte count was 14,600, erythrocytes 4.1 million, and hemoglobin 80 per cent Sahli. The Kline exclusion test was negative. Blood cultures gave no growth. The blood urea nitrogen on admission was 15 mg. per 100 c.c. Sputum typing yielded a type XI pneumococcus. X-ray examination of the chest showed what was interpreted as bronchopneumonic infiltration in both lower lobes.

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The patient was given 4 Gm. of sulfapyridine during her first hospital day (April 30). The following morning her temperature was normal and the drug was discontinued. While her urinary output at this time was not measured accurately, it appeared to be adequate.

On May 2, the third hospital day, the temperature rose to 100.4° F. and the administration of sulfapyridine was resumed. During the next thirty-six hours the patient received a total of 4 Gm. in 0.5 Gm. doses. The drug was then discontinued.

On May 3 the temperature fell to normal, the pulse was 80, and the respirations 22. On this day, however, the patient complained for the first time of abdominal pain; she was unable to void. The next day 45 c.c. of grossly bloody urine were obtained by catheter. The blood sulfapyridine was 1.8 mg. per 100 c.c., and the blood urea nitrogen was 20 mg. per 100 c.c.

On May 5 the total output of urine was only 6 c.c. This was bloody and was obtained by catheter. The blood urea nitrogen had increased to 42 mg. per 100 c.c. Despite this her pneumonia showed signs of resolution. One intravenous infusion of 300 c.c. of physiologic saline and 100 c.c. of 50 per cent sucrose were given.

During the next three days (May 6, 7, and 8) only 15 c.c., 2 c.c., and 86 c.c. of urine, respectively, were obtained by catheter. All the specimens were grossly bloody and contained large traces of albumin. Specific gravities corrected for protein varied from 1.010 to 1.019. The patient received several infusions of physiologic saline and glucose. On May 6 the blood pressure, which had previously been about 165 systolic and 80 diastolic, rose to 190 systolic and 90 diastolic.

The patient died of uremia on May 8, the ninth hospital day and five days after the administration of sulfapyridine had been discontinued. She showed no convulsions. The blood urea nitrogen on the day of her death was 104 mg. per 100 c.c. The final clinical diagnoses were toxic glomerulonephritis due to sulfapyridine and uremia.

*Autopsy* was performed two hours after death. It was limited to the abdomen, but examination of the thoracic organs through the abdominal incision revealed that the pneumonia was either completely resolved or minimal. The heart appeared to be enlarged.

The essential pathologic findings were in the urinary tract. Each kidney weighed 260 Gm. and was the seat of slight arterial and arteriolar vascular disease. Cross section showed marked bulging of the parenchyma, which was pale gray in color; the usual striations in the cortex were obscured. The pelves were of normal size and shape but were lined by a thick, edematous, grayish-brown mucosa which showed irregular hemorrhages extending into the adjacent peripelvic fat. The mucosa of the minor calices and of the renal papillae was greenish-brown and necrotic and presented a ragged surface covered by deposits of granular and amorphous reddish-brown debris. Chemical analysis of this material for sulfapyridine and its derivatives was negative.

The ureters showed no dilatation. In their upper portions the mucosa was thickened and showed many small areas of hemorrhage. However, the distal 4 cm. of each ureter, including the portions traversing the wall of the bladder, revealed a swollen and necrotic, greenish-brown mucosa, and the lumina were markedly reduced. The bladder showed diffuse edema and focal hemorrhages of the mucosa.

Microscopically, in addition to slight arteriolar sclerosis, the kidneys were the seat of a marked nephrosis. The epithelial cells of the tubules were enlarged and presented a pale-staining, foamy, or transparent cytoplasm, and very distinct cell borders. The nuclei were of usual size and showed no necrotic change. The swelling of the cells had resulted in moderate diminution of the lumina of the tubules. The glomeruli showed no significant change. There was no inflammatory exudate.

Microscopically the renal pelves showed marked edema of the mucosa, foci of polymorphonuclear and lymphocytic infiltration, and focal hemorrhages extending into the adjacent peripelvic fat. The lining of the minor calices and of the renal papillae was necrotic and ulcerated, and partly replaced by masses of blue and greenish-brown necrotic material. This lesion was associated with dense infiltrations of polymorphonuclear cells. The upper portions of the ureters showed a slight degree of inflammation, edema, and focal hemorrhage.

the terminal portions extending to the orifices in the urinary bladder showed the same type of necrotizing and suppurative inflammation present in the renal calices. The urinary bladder was the seat of an acute hemorrhagic cystitis and showed polymorphonuclear and lymphocytic infiltration of the mucosa.

The liver, spleen, and gastrointestinal tract showed no significant gross or microscopic change.

The final pathologic diagnoses were: acute necrotizing and purulent pyelitis and ureteritis with hemorrhage; marked acute nephrosis; acute hemorrhagic cystitis; minimal arterial and arteriolar nephrosclerosis.

#### COMMENT

Although this patient was admitted to the hospital with a bilateral bronchopneumonia, she did not appear to be seriously ill. On admission her urine was negative, her urinary output was satisfactory, and her blood urea nitrogen was only 15 mg. per 100 c.c. In the treatment of her pneumonia she received a total of 8 Gm. of sulfapyridine, of which 4 Gm. were given on her first hospital day and 4 Gm. on her third and fourth hospital days. She was apparently making an uneventful recovery until the fourth hospital day, when she complained for the first time of abdominal pain and inability to void. Catheterization the following day yielded 45 c.c. of grossly bloody urine. During the next four days, although her pneumonia appeared to be resolving, she did not void spontaneously, and a total of only 109 c.c. of bloody urine were obtained by catheter. Blood urea nitrogen determinations were 30 mg. per 100 c.c. on May 2, 42 mg. on May 5, and 104 mg. on May 8, the day of death. Death occurred eight days after sulfapyridine therapy was started.

At autopsy the essential pathologic findings consisted of an acute necrotizing, purulent, and hemorrhagic pyelitis and ureteritis, and a marked acute nephrosis. The inflammatory lesion was especially severe in the minor calices and renal papillae and in the terminal portions of the ureters which were obstructed as a result of mucosal swelling, necrosis, and exudate. The lesions appeared to be of short duration; there was no evidence of chronicity. Although no calculi were found in the urinary tract, there were extensive deposits of reddish-brown granular material lining the pelvic surface of all the renal papillae. Chemical analysis of this material for sulfapyridine and its acetyl derivative was negative. There was no inflammation within the kidney.

Similar lesions of the urinary tract associated with the formation of uroliths, consisting of the acetyl derivative of sulfapyridine, have been produced experimentally in animals by means of the oral administration of the drug.<sup>1, 2</sup> Antopol and Robinson<sup>1</sup> noted the formation in the urinary tracts of susceptible rats, rabbits, and monkeys of aggregates of needlelike crystals, especially in the ureters and most frequently at the level of the bony pelvic brim. These lesions were often associated with more or less complete urinary obstruction. The early picture in the urinary tract was that of calculous ureteritis and pyelitis; with involvement of the kidney a pyelonephritis ensued.

Hematuria has also been reported as a complication of sulfapyridine therapy in man. Southworth and Cooke<sup>3</sup> recently described three such patients, two of whom had severe renal and ureteral pain, while two had nitrogen retention.

All recovered promptly when the drug was stopped and adequate fluids were given. From the experimental and clinical evidence available thus far, it would appear that sulfapyridine is capable of producing a hemorrhagic and suppurative inflammation of the urinary tract, especially of the ureters and renal pelvis, associated with necrosis and formation of concretions. The latter consist of sulfapyridine and its acetyl derivative. Possibly the concretions are formed first, and the inflammatory lesions are secondary. The attendant oliguria and pain are presumably due to the obstruction and urinary stasis in the pelvis or ureters caused by the inflammation, the calculi, or both these factors.

Both the clinical course and the autopsy findings in the present case justify the belief that the sulfapyridine was the etiologic agent responsible for the urinary tract lesions. Prior to the use of the drug the patient's urine was negative and her urinary output was adequate. Following the administration of 8 Gm. of sulfapyridine there was rapid development of hematuria, oliguria, and azotemia. Except for small doses of codeine, no other drugs were given. The patient received one infusion of 100 c.c. of 50 per cent sucrose, but this was administered after the onset of the oliguria and hematuria. The necrotizing and hemorrhagic type of lesion in the pelvis and ureters indicates a toxic and irritative injurious agent of chemical rather than bacterial nature.

The marked tubular disease present in the kidneys may be due largely, or in part, to the sucrose which the patient received shortly before death, rather than to the sulfapyridine. Nephrosis has been observed at autopsy in patients who received sucrose during life.<sup>4</sup> Similar tubular damage has also been produced in animals by the intravenous administration of sucrose, although the amounts used were comparatively large and the nephrosis was not associated with renal insufficiency or with inflammation in the urinary tract.<sup>5</sup> That the sucrose is not the primary injurious agent in this case is indicated conclusively by the fact that it was used after the onset of hematuria and oliguria. The patient's pneumonia was minimal and in the process of resolution and was in all probability not related to the fatal outcome or to the disease in the urinary tract.

#### SUMMARY

A case of fatal renal insufficiency incident to the administration of sulfapyridine is described. The patient was an 80-year-old woman who received a total of 8 Gm. of the drug over a period of three days in the treatment of a bronchopneumonia. She then rapidly developed abdominal pain, hematuria, and marked oliguria. During the next five days a total of only 154 c.c. of bloody urine was obtained by catheter. Death occurred in uremia eight days after the onset of administration of the sulfapyridine and five days after it was discontinued. At autopsy there was a severe necrotizing, suppurative, and hemorrhagic ureteritis and pyelitis, with obstruction of the terminal portions of both ureters, and a marked acute nephrosis.

Grateful acknowledgment is made of aid rendered by Dr. Joseph M. Hayman, Jr.

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## THE EFFECTS OF INSULIN ON SERUM LIPIDS AND CHOLINE ESTERASE IN SCHIZOPHRENIA\*

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ALTHOUGH we were unable to demonstrate any consistent immediate effect of insulin on the postabsorptive level of the whole blood lipids in a previous study,<sup>12</sup> we observed a mild lipemia on repeated treatments. After cessation of treatment the lipids remained elevated in those patients showing remission but tended to return toward the initial level in the unimproved patients. Since serum lipids are more susceptible to change under the influence of extraneous agents than are whole blood lipids, we studied the immediate effects of insulin on the postabsorptive level of serum lipids as well as the chronic effects of repeated doses in another series of patients. Hematocrits were included to control variations in serum concentration, an important factor in insulin coma because of the severe perspiration. Since insulin has been suspected of exerting a stimulating effect on the sympathetic nervous system<sup>5</sup> and since it has been postulated that the choline esterase activity of serum is related to the autonomic balance,<sup>7</sup> we have included this variable as a corollary to the study of lipid variation coincident with variation of autonomic activity.

### METHODS

The prolonged effects of the insulin hypoglycemia treatment on serum lipid, choline esterase, and hematocrit were studied in 17 schizophrenic patients. Two blood samples (a week apart) were taken before beginning treatment, another during the fourth week of treatment, and two more (a week apart) after cessation of treatment. In order to study the immediate effect of insulin, further samples were taken on 12 patients (in the fourth week of treatment) in the postabsorptive state, during the period of coma, and one hour after the administration of sugar which was used to terminate the coma. Serum phospholipid and total lipid were determined in duplicate by Bloor's oxidimetric methods.<sup>2</sup> Free and total cholesterol were determined by Turner's modification of the Okey digitonide method.<sup>17</sup> Choline esterase activity was determined by Ammon's gasometric method.<sup>1</sup> The relative volume of serum to cells was determined after centrifuging the heparinized blood in a graduated tube.

\*From Research Service of Worcester State Hospital, Worcester.

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## RESULTS

In Table I are presented the means and criteria of significance of differences of serum lipids, choline esterase, and hematocrit obtained before treatment, during the fourth week of treatment, and after cessation of treatment. It is apparent that the various lipid fractions and choline esterase reach the maximum values during the midcourse of treatment. With the exception of free cholesterol the mean values are significantly lower at the end of treatment than those in midcourse, but they are significantly higher than the pretreatment means.\* It is noteworthy that the hematocrit value did not change significantly during the course of treatment.

TABLE I

SHOWING THE DIPHASIC COURSE OF SERUM LIPIDS AND CHOLINE ESTERASE IN CONTINUOUS INSULIN TREATMENT\*

VARIABLE	N	MEAN VALUES			SIGNIFICANCE OF DIFFERENCE IN TERMS OF THE "T" CRITERION		
		BEFORE TREATMENT	IN MID-COURSE OF TREATMENT	AFTER TREATMENT	BEFORE TREATMENT VS. MID-TREATMENT	MIDTREATMENT VS. AFTER TREATMENT	BEFORE TREATMENT VS. AFTER TREATMENT
Total lipid mg./100 c.c.	17	402.7	546.2	449.6	4.9*	3.1*	2.96*
Phospholipid mg./100 c.c.	17	177.4	231.6	202.3	4.6*	2.5†	3.08*
Total cholesterol mg./100 c.c.	17	159.8	192.5	173.8	4.7*	3.0*	2.69†
Free cholesterol mg./100 c.c.	17	49.1	54.9	46.5	1.8	3.1*	0.46
Choline esterase c.mm./hr./c.c.	14	68.2	82.1	80.0	6.04*	1.17	3.04*
Hematocrit per cent	17	44.4	44.4	44.2	--	--	--

\* $P < 0.01$ .

† $0.05 > P > 0.01$ .

The behavior of serum lipids and choline esterase in the midcourse of treatment was exceptionally uniform from individual to individual. The rise in lipid level occurred in all cases, and choline esterase rose in all cases except one, in which there was no change. There occurred considerable variation among different patients in the values obtained during the two weeks following the treatment. These data were discussed in a separate paper<sup>13</sup> in which it was shown that the serum lipids remained elevated in those patients who improved coincident with the treatment but returned toward the pretreatment level in those patients who remained unchanged mentally.

In Table II are presented the means and criteria of significance of differences for serum lipids, choline esterase, and hematocrit in 12 patients before and during the course of a single insulin treatment. Although for all the variables we find a rise in the mean level above the postabsorptive level and a tendency to drop again after the administrations of sugar, only the trends for choline esterase and hematocrit are statistically significant. The behavior of lipids during coma, and especially on administration of sugar, varied greatly

\*The significance of differences was determined through "t" criteria.

from individual to individual. In 8 of the patients the lipids rose to greatly varying degrees, while in 4 patients there was a fall or no change.

All patients except one showed a rise in choline esterase and all showed the increase in hematocrit during coma. The question arises whether or not the increased blood concentration accounts for all the increase in choline esterase. The intra-individual correlation coefficient, obtained on the control and coma values of hematocrit and for choline esterase, was 0.67. Thus the hematocrit change accounted for 26 per cent of the variation of choline esterase. The larger proportion of the variation in choline esterase may, therefore, be ascribed to factors other than hemococoncentration. Since the mean hematocrit change was 5.8 per cent and that for choline esterase was 12.5 per cent, we believe that insulin produced a significant rise in choline esterase.

TABLE II

SHOWING MEAN CHANGES IN SERUM LIPIDS, CHOLINE ESTERASE AND HEMATOCRIT DURING INSULIN COMA AND AFTER ADMINISTRATION OF SUGAR

VARIABLE	N	MEAN VALUES			SIGNIFICANCE OF DIFFERENCES IN TERMS OF THE "T" CRITERION
		CONTROL	COMA	AFTER SUGAR	CONTROL MEAN VS. COMA MEAN
Total lipid mg./100 c.c.	12	554.6	599.2	585.9	2.37
Phospholipid mg./100 c.c.	12	229.0	237.0	228.4	1.16
Total cholesterol mg./100 c.c.	12	192.3	203.9	191.1	2.19
Free cholesterol mg./100 c.c.	12	53.7	58.0	57.3	1.46
Choline esterase c.mm./hr./c.c.	10	84.8	95.5	87.7	4.30*
Hematocrit per cent	12	44.43	47.09	46.60	5.99*

\* $P < 0.01$ .

## DISCUSSION

The variable response of the postabsorptive level of the serum lipids among different individuals indicates that insulin acts only indirectly on the serum lipids, perhaps through a disturbance in the carbohydrate or acid-base balance. Reports of other workers on the effect of insulin on the postabsorptive level of blood lipids are equally inconsistent. In normal men Christomanos<sup>4</sup> reported a slight fall, while Bruger and Mosenthal<sup>3</sup> found no consistent effect. In animals a drop in blood lipids was reported by Page, Pasternak, and Burt,<sup>11</sup> Schmidt and Ssaatchian,<sup>15</sup> Himwich and Spiers,<sup>6</sup> and Wertheimer;<sup>18</sup> no change was indicated by the results of Rony and Ching<sup>14</sup> and Miller;<sup>10</sup> and a rise was found by White.<sup>19</sup>

There is little reason for doubting that insulin lowers an abnormally high blood lipid level, although this is likely to be due to an indirect effect of insulin on carbohydrate metabolism. Treatment of the lipemic diabetic person with insulin lowers the blood lipid in both human subjects<sup>8</sup> and dogs.<sup>9</sup> Insulin causes a phlorizin lipemia<sup>18</sup> or nephrotic lipemia<sup>16</sup> to disappear. On the other hand, we have found a prolonged rise in serum lipids after insulin administration in patients who have an abnormally low serum lipid level.<sup>13</sup> The daily injection

of large doses of insulin appears to raise the lipids toward the normal level, which is maintained after cessation of treatment only in those patients who improve mentally coincident with the treatment. The mean choline esterase level of the patients did not differ significantly from that of normal subjects. The insulin therapy produced a rise in choline esterase in all patients, followed by a return toward the initial level without regard to the clinical progress of the patients.

## SUMMARY

During the course of treatment of 17 schizophrenic patients with large daily doses of insulin, the serum phospholipid, total cholesterol, free cholesterol, total lipid, and choline esterase rose above the pretreatment level. These values remained elevated for two weeks following treatment.

In 12 patients studied during the fourth week of treatment insulin did not produce any consistent immediate change in serum lipids, but it increased the choline esterase to a significantly greater extent than could be accounted for by hemoconcentration.

I am indebted to Mr. E. B. Romanoff for some of the choline esterase determinations.

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## VARIATION OF BLOOD PRESSURE WITH BRIEF VOLUNTARY MUSCULAR CONTRACTIONS\*

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IN A PREVIOUS study<sup>2, 3</sup> the blood pressure of various subjects was taken from time to time during periods of from one to two hours, while graphic records were made concerning their states of skeletal muscle tension.† Determinations were made of systolic as well as of diastolic pressure in some patients with vascular hypertension as well as in subjects of normal type. Skeletal muscle tension was measured in terms of action potentials, employing the right biceps-brachial muscle group in all instances, along with one or more other groups in most instances. In order to eliminate the influence of change of posture to the lying position, no reading of blood pressure was considered until the subject had been lying for at least fifteen minutes.

The evidence pointed clearly to a relationship in man between the state of contraction in the manifold muscle groups of the individual and his blood pressure at a corresponding instant. This accords with an extensive literature on physical exercise, which it seems unnecessary to review here, but from which it is well known that during vigorous muscular contraction, the blood pressure rises, at least until fatigue sets in; this applies to systolic, if not always to diastolic, pressure. However, one writer (Fishberg,<sup>4</sup> 1934) finds that slight muscular exercise produces little or no change in the blood pressure. In any event, the relationship between contraction in divers skeletal muscle groups and variations in blood pressure is not simple, and is readily obscured in the presence of other factors that influence the blood pressure. More evidence is needed to confirm the existence of this correlation and to clear up our conceptions about it.

The impression has been gained that under conditions kept fairly constant otherwise, a marked change in the state of general muscle tension is likely to be accompanied by a shift in the blood pressure level. Blood pressure tends to fall with progressive muscular relaxation, especially as this nears completion; but after lower levels of pressure are once established, merely prolonging the rest, without increasing the degree or extent of relaxation, does not necessarily effect an additional fall in pressure. Likewise, if from the outset there is prolonged tension in the muscle groups studied during

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Mr. William M. Lees assisted during these experiments.

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†By tension is here meant active contraction, not passive stretch.

the rest period, there is not necessarily a marked rise of pressure; if general muscle tension fluctuates fairly constantly within certain limits, the blood pressure may show little variation during the period.

While it is certain that skeletal muscles do not necessarily relax fully during a period when an individual attempts to rest,<sup>5</sup> this is not generally realized as yet, and there is still the tendency in many quarters to identify lying down with relaxation. The influence of posture can be eliminated most effectively if, while keeping this constant during an experimental period, we produce alternately contraction and relaxation in selected muscle groups, and determine whether there occur corresponding increases or decreases in blood pressure. To effect this in the present studies, the subject, while lying at rest in other respects, clenches his right fist for several minutes, generally about five, sometimes up to ten.

Although the studies have been made chiefly in the lying position, similar results are observed if the subject sits up in a fairly comfortable chair. In that position a certain degree and extent of relaxation commonly occurs under normal conditions, as has previously been shown by various methods. For example, the knee jerk, responding to an intermittent stimulus of constant magnitude, shows a characteristic decline in extent in many instances.<sup>6</sup> Furthermore, an individual may be seated but so relaxed that a continuous record of zero activity, or nearly so, is shown from the muscles of either arm. In short, while sitting, relaxation may be more or less complete in muscle groups whose activity is not required for that posture.

As stated previously, preliminary studies of several hypertensive patients in the sitting posture indicated that the blood pressure often rose when they contracted the muscles in a part (such as the upper arm) during a test period of about ten to sixty seconds, falling subsequently if they then relaxed those same muscles while remaining in the sitting posture. The customary method of combined palpation and auscultation was employed. Such procedures in studying rise and fall of pressure obviously enable us to rule out the influence of posture, since this continues constant; they also keep emotional factors down to a minimum of change.

Observations can be made more precisely if muscle tensions are measured in terms of action potentials. Two amplifier string galvanometer assemblies were employed, recording action potentials, as stated previously, to a fraction of a microvolt. For this, 15 subjects were available. The blood pressure was normal in 11, 6 of whom had received training to relax, while the other 5 were athletes. The 4 remaining subjects showed chronic high blood pressure and were being trained to relax. All subjects had participated in previous experiments, and accordingly, were well accustomed to the conditions of test. In the following studies, the subject, as a rule, reclines and has been instructed to clench his fist continually and vigorously, beginning when he hears a telegraph key click near his ear. The moment of this click is indicated on the photographic records by the movement of the pointers of two Duprez signal magnets—one placed before each camera. A second click recorded similarly is the

signal to cease a requested muscular contraction, such as clenching. Accordingly, the time relations can be charted accurately. In order to eliminate subjective errors in determining blood pressure, the Tyco's self-recording sphygmomanometer is again employed in all but the early records in this series. As said previously, the point of systolic pressure is sharply indicated on the charts made with this instrument, but there is question at times concerning the reading of the diastolic pressure. Accordingly, we may believe that the figures for systolic pressure have a negligible error, but no such assumption is made regarding those for diastolic pressure. However, since all readings were made by two persons in association, we may assume that errors, if any, were in the same direction, and that the results for diastolic pressure are satisfactory at least for purposes of comparison. In any event, the general character of the results on diastolic, as well as on systolic, pressure does not seem to differ very much whether the automatic apparatus is used or the ordinary method of palpation plus auscultation.

To determine and evaluate the influence on blood pressure of the voluntary contraction of selected muscle groups, it is necessary to keep other factors as nearly as possible constant. Accordingly, in the present experiments, noises and other disturbing factors are avoided. It was evident that the subjects were not in an emotional state. The instruction to contract certain muscle groups, while keeping others as relaxed as possible, was probably carried out more precisely by the subjects trained to relax; but the athletes were skillful at this as well.

While the results to be presented are not positive in all instances, they seem to reveal a trend distinctly. The type of result often secured in a subject with normal blood pressure upon prolonged complete or approximately complete relaxation interrupted at intervals by muscular contractions, such as clenching the right fist, appears in Fig. 1.

Ro. was a young woman, 24 years old, who had recovered from a condition of severe nervous depression, *pari passu* as she learned to be relaxed. She readily achieves marked relaxation in the right arm (*biceps brachialis*) and the left leg (*quadriceps femoris*) muscles in which potentials are measured; the voltages generally are near 0 microvolt, but do not exceed 1 microvolt. She is somewhat less successful in relaxing the abdominal muscles, and still less those of the eyes. On the whole, the degree of relaxation attained is sufficient for present purposes.

About fifteen minutes after lying down, the first blood pressure is taken, whereupon recording of action potentials is begun, while she relaxes fairly for about fifteen minutes more. Upon firmly clenching the fingers of the right hand, there occurs promptly an increase in systolic pressure (8 mm.) and in diastolic pressure (5 mm.). In this and in similar figures considered here, the blood pressure readings during any contraction will be set down as positive or negative, as compared with the last reading before the contraction. (An alternative procedure, but leading to practically similar results, would be to take the mean of the readings prior to the contraction.) This increase is not maintained in the second blood pressure reading toward the close of the five-minute period of clenching, where the values are but  $+4/-1$ , as compared with the reading prior to the contraction. During this same five-minute interval, action potentials from the right arm muscles decline somewhat in voltage, showing that the patient has not maintained the clenching of the fist steadily; possibly this explains in part

the decline in pressure during the same period. Upon clenching the second time, the rise in pressure is more marked than before (12/8), while the second reading toward the close of the five-minute period of clenching is 12/16. Evidently, there is no decline in pressure during the five-minute period of clenching this time, possibly because the abdominal muscles contracted somewhat more during this period, producing an additive effect on the pressure.

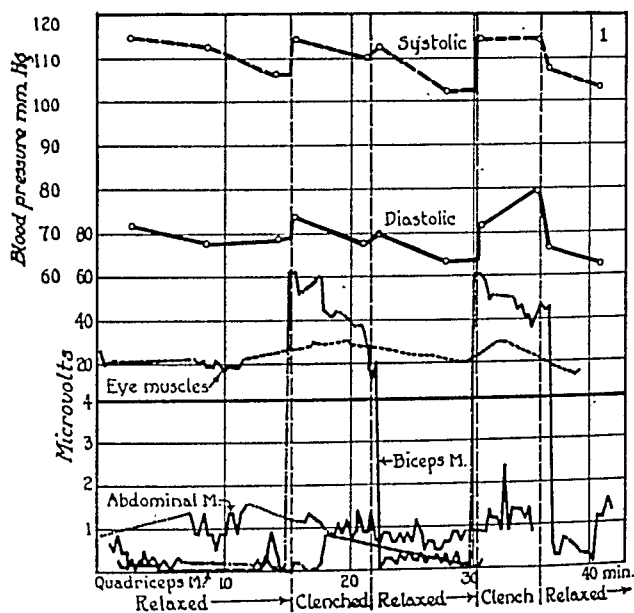


Fig. 1.—Showing rise of systolic and diastolic pressures during clenching of the right fist in a woman aged 24, trained to relax fairly. Plotted against time are systolic and diastolic blood pressures in millimeters of mercury, but also microvoltage of action potentials from certain muscles in the right arm, left thigh, abdomen, and about the eyes (perhaps including retinal potentials).

As described previously, the cameras do not operate continuously and, in consequence, records of action potentials are not complete for four leads. Accordingly, interpolation appears on this graph as well as in Figs. 3, 4, and 5.

In the 11 subjects with normal pressure, 70 determinations in all were made during clenching of the right fist. Systolic pressure was increased in 64, and diastolic pressure was increased in 63 of these determinations.

A composite graph for these 70 determinations appears in Fig. 2, covering a time period of ten minutes. During clenching of the right fist in these subjects there occurs, on the average, a significant rise in systolic and diastolic pressures up to at least ten minutes. There is some indication of a tendency for diastolic pressure to accompany systolic pressure in its rises. (In most instances clenching was not prolonged after about five minutes.) In all instances readings were not made each minute, since inflating the cuff so often would inevitably influence the results. Accordingly, no conclusions are warranted from the character of the curve shown in Fig. 2, except that, in general, the results are strongly positive.

Fig. 3 furnishes an illustration of a patient suffering from vascular hypertension of advanced type.

L., a matron of about 37 years, was referred for study from the Billings Hospital through the kindly cooperation of Doctors Franklin McLean and Louis Leiter. The diagnosis



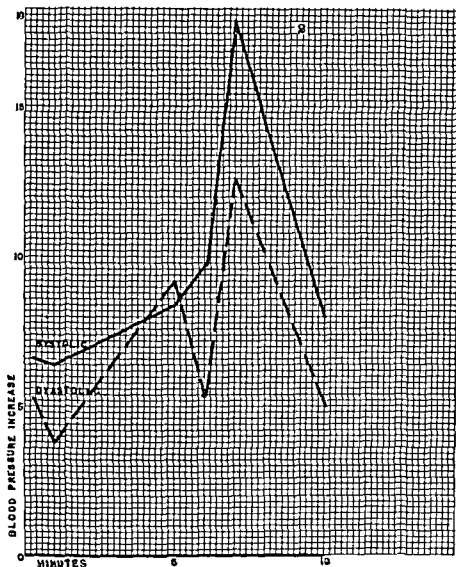


Fig. 2.—Rise in systolic and in diastolic pressure during protracted clenching of the right fist. Composite graph of 70 determinations in 11 subjects with normal pressure.

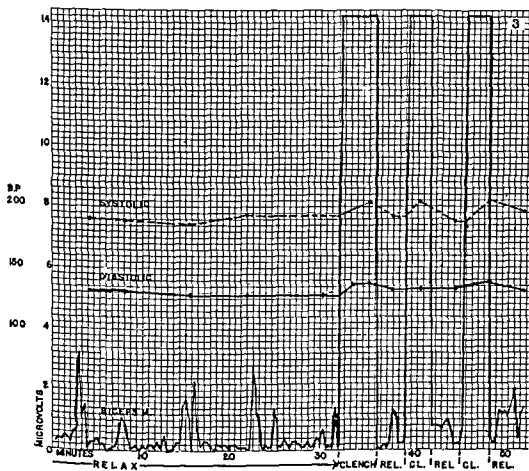


Fig. 3.—Plotted against time are systolic and diastolic blood pressures, as well as action potentials from the flexors in the right upper arm. Rises in systolic and diastolic pressures generally mark the intervals in which she clenches her right fist as contrasted with the periods in which she does not.

was hypertension, with evidences of advanced renal pathology. Action potentials from the flexor muscles located in the right upper arm are plotted against time. As will be noted, her relaxation is far from perfect. After about two minutes of clenching the right fist, pressure rose in the amount of 5/9, and about three minutes later the increase became 11/10. During the five-minute period, when clenching ceases and relative rest is resumed, the pressure drops back approximately to the levels which preceded the act of clenching; but two minutes after clenching has been resumed once more, pressure again increases in the amount of 11/1. Decline of pressure occurs again during the following control period. During the third test period of clenching, the pressure rise after about four minutes is 17/5. Thereafter, the pressure declines again approximately to former resting levels.

The data on four subjects with hypertension are too few to graph, but the results are roughly on the same order as for those with normal pressure. At one minute the average rise is 5.7/4.3; at five minutes it is 8.3/5.7; at ten minutes it is 9.7/8.0. Upon voluntary contractions, while lying, we should expect greater increase in blood pressure—in millimeters of mercury—during states of chronic high blood pressure than during normal states. Our findings, however, show the greatest increases, as a rule, in three subjects whose pressure levels are normal. The explanation probably is that these were athletes: they obviously clenched their fists more tightly and vigorously than did the subjects in the other groups. This observation is readily confirmed upon reading the voltages from muscles in the arm during clenching, which are found to exceed considerably the similarly recorded voltages in the trained subjects (not athletes) and in the subjects with abnormally increased blood pressure. It seems safe to conclude that the more vigorous contraction in the normal athletes accounts for the greater increase in pressure observed in the group of athletes as compared with the hypertensive group.

In all subjects more strongly positive results are often secured if additional muscle groups participate in the voluntary contraction. This is well illustrated in Fig. 4.

The subject is a professor in a medical department, aged 37 years, whose original complaints were from spastic colitis of thirteen years' duration; there were no symptoms of cardiovascular origin. At the time this record is taken recovery was evidently complete, and his technique at relaxation, as shown in Fig. 4, was adequate. After lying down for about twenty-five minutes, the pressure reading was 118/69. Upon clenching his right fist vigorously, the pressure taken at once was 122/76, an increase of 4/7. When clenching had been protracted for five minutes, the reading became 136/81, a total increase of 18/12. Upon stiffening both legs, but also continuing to clench the fist, the pressure taken at once was 145/86, a total increase of 27/17; when protracting these contractions for five minutes, the reading became 147/87, a total increase of 29/18. When instructed to relax once more, the reading fell at once to 127/74—a return approximately to the levels present prior to the original clenching. From this level, subsequent clenching of the right fist produces a rise in systolic and in diastolic pressures to a peak increase of 11/9—a figure less than half of the peak increase noted during the joint contraction of right arm and both leg muscles, namely, 29/18.

Augmentation of blood pressure in the lying position occurring upon increased involvement of skeletal muscle groups (progressive muscular tension) is further illustrated in Fig. 5.

An athlete, aged about 20 years, shows normal pressure. After lying quietly for almost fifteen minutes, the record begins with right arm muscles recorded relaxed perfectly, while

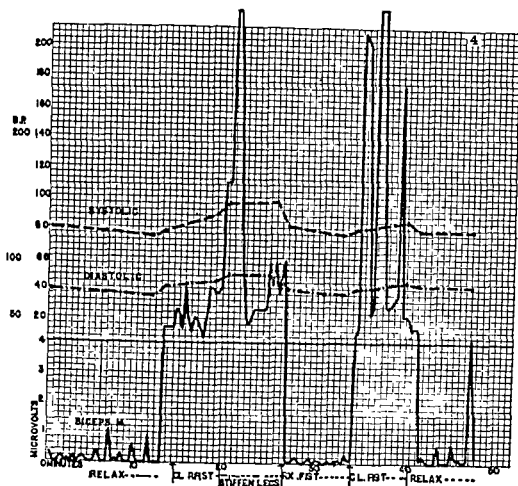


Fig. 4.—Systolic and diastolic pressures, as well as action potentials from the flexor muscles in the right upper arm, plotted against time. Subject is trained to relax; blood pressure is normal. The rise in pressure occurring upon clenching the right fist is augmented, both systolic and diastolic, upon additional voluntary contraction in both legs

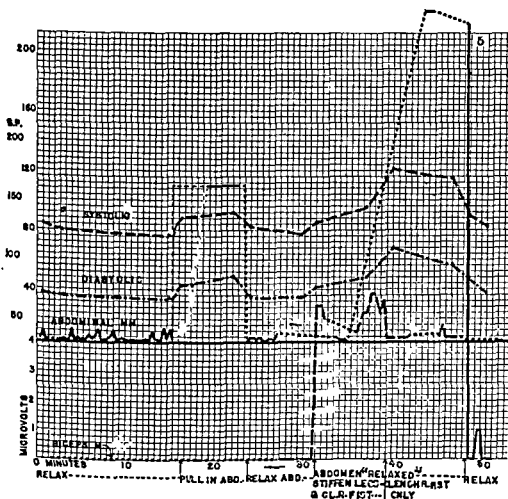


Fig. 5.—Plotted against time are systolic and diastolic blood pressures, as well as action potentials from the flexor muscles in the right upper arm and from abdominal muscles. A sustained rise in systolic and diastolic pressures occurs with continued contraction in the abdominal group. Pressure returns to resting levels upon muscular relaxation. When the right fist is clenched and the legs stiffened as well, the pressure rise is very definite; but it becomes greatly augmented when, contrary to instruction, the abdominal muscles are contracted in addition. Upon relaxation, pressure returns to former resting levels.

the electrodes in abdominal muscles reveal over 4 microvolts of potential differences. The pressure rises from 120/67 to 135/78 upon "pulling in" the abdominal muscles, and at the end of five minutes (while the arm muscles continue to be relaxed) the pressure reaches 140/86—a total rise of 20/19. Upon relaxing the abdomen to former levels, a fall occurs promptly to 128/68, and after five minutes of continued relaxation, the blood pressure has returned to former resting levels, 122/68. The instruction to clench the right fist and to stiffen both legs is followed promptly by a rise to 131/77, and when this is continued for five minutes, the pressure increases to 144/85, a total increase of 22/17. In addition, but contrary to instruction, he pulls in the abdominal muscles, whereupon the pressure rises to 176/110, a total increase of 54/42. While these various muscle groups continue in sustained contraction, there occurs at the end of five minutes a fall to 168/95, reducing the total increase at this point to 46/27. Upon instruction, the abdominal muscles now relax perfectly for the first time in this record, but the right arm remains somewhat tense; thereupon blood pressure falls to 137/84, and after five minutes to 128/72, which is 8/5 higher than before the original voluntary contraction of the abdominal muscles.

Increased blood pressure following upon "pulling in" the abdominal muscles may be due, in part at least, to pressure upon abdominal (splanchnic) blood vessels. Evidence to this effect will be considered in a later article. However, this is probably not the full explanation, for little or no such pressure on the abdominal blood vessels occurs if the subject is instructed to make the abdominal muscles rigid but without squeezing inwards, or if he is instructed to push out the abdominal wall by muscular effort; yet these alternatives are accompanied by rise of blood pressure in various instances recorded during the present studies.

When the subject, lying down, engages in a voluntary contraction following a period of attempted relaxation, a rise of pressure is not always clearly notable by ordinary methods of measurement. Sometimes the reasons for such negative results are obscure; at other times the interpretation seems fairly evident. A negative instance with the subject L, which can be compared with Fig. 3, follows:

She fails to relax when requested to do so. Action potentials from the right arm muscles are relatively very high before as well as after she clenches her fist, frequently and repeatedly reaching 7 microvolts or more. Toward the end of the initial period of unsuccessful attempts to relax, the pressure is 208/120. Not an increase, but a drop to 197/118 occurs after five minutes, during which she has been clenching her right fist. After five minutes more of this activity, the pressure has risen to 213/122. She ceases to clench her fist, and returns once more to attempts to relax which prove unsuccessful; after intervals of five and eighteen minutes, the readings are 219/119 and 210/118, respectively. In short, blood pressure is not higher during the period of clenching than during the period of attempted relaxation, apparently because she fails to relax. In the relaxing periods shown in Fig. 3, she was considerably more successful.

Accordingly, this instance illustrates once more that the attempt to relax, if unsuccessful, does not characteristically bring with it a fall of blood pressure, as compared with a foregoing period during which contraction has been marked.

Evidently, if a muscle group has been in a state of marked contraction, a moderate additional contraction therein may be insufficient to make itself apparent in a change in the recorded blood pressure. Presumably it is the relative degree of augmentation in contraction (not the absolute amount, measured in dynes, calories, or microvolts) which shows up in the results.

A generalization here, if data were sufficient to permit this, could be expressed in terms analogous to Du Bois-Reymond's law applied in the neuromuscular field. This accords also with the comments made previously that after contraction has subsided so that it is minimal, augmentations in contraction are accompanied by more obvious rises in blood pressure.

#### SUMMARY AND CONCLUSIONS

Experiments are conducted on 11 subjects with normal blood pressure and on 4 with chronically high blood pressure, all of whom relax at least fairly well upon request, whether through special training or otherwise. Electrical measurements are taken of contraction (action potentials) in various muscle groups, while blood pressure is recorded at certain times from the left upper arm.

In an individual otherwise relaxed, successive contraction of a muscle group or groups (for example, clenching one fist) for periods of several minutes or more, and then relaxing completely for similar periods, is frequently accompanied by corresponding rises and falls of systolic and diastolic pressures. The extent of the relative rise and fall depends not only upon the intensity of the particular voluntary contraction but also upon the degree and extent of relaxation present in the general musculature. No contrasting rise in pressure may be evidenced if the patient is not well relaxed before he clenches his fist. The addition of marked contraction in other regions (e.g., if he contracts muscles in the lower limbs or elsewhere at the same time as he clenches his fist) promotes further rise in blood pressure.

Under the conditions of the experiment, it is evident that emotional factors are reduced to a minimum or absent altogether. At the very least it seems safe to say that no more emotion is present when the subject clenches his fist or stiffens his legs than when he tries to relax. Evidently, therefore, emotional variations do not account for the variations in pressure which correspond with variations in skeletal muscle contraction. The indications are that progressive tension in a muscle group tends to elevate systolic and diastolic pressures, and that this tendency increases if contraction spreads to other groups. Accordingly, the present findings lend further support to the view that in individuals with normal, as well as with increased, pressure (essential hypertension), the pressure levels at any instant vary to an important and determinable extent with the magnitudes of contraction in the various skeletal muscles.

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## THE TREATMENT OF EDEMA BY RECTAL ADMINISTRATION OF DIURETICS\*

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THE intramuscular and intravenous administration of mercurial diuretics has become widely accepted as a reliable treatment for the edema of congestive heart failure. It seems generally agreed that undesirable systemic or local reactions or subjective symptoms occur infrequently and are seldom of sufficient severity to contraindicate their use. In recent years mercurial diuretics have been applied in the form of suppositories, with the view of rendering their administration more convenient. It is the purpose of this report to review the literature on mercurial diuretic suppositories to date and to evaluate the present status of such preparations for the treatment of edema. Results of additional clinical studies will be included.

Apparently the first report on this method of administration of mercurials was published by Natanson<sup>1</sup> in 1930. In this study, salyrgan, which had been used parenterally for several years, was applied rectally in a glycerin base. In 1934 Engel<sup>2</sup> reported on the use of mercurin† in a cocoa butter base. Only these two mercurial diuretics have been available as suppositories up to the present time. Subsequent investigations have been concerned exclusively with the mercurin suppository. In Table II a summary is given of those papers of which the data are reasonably complete.

All observers have found that the mercurin suppository produces a definite diuresis, comparable to that resulting from the intravenous administration of mercurials, although in most cases quantitatively less. Thus, Thomson<sup>3</sup> obtained an average urinary output of 2,360 c.c. on the day following the administration of suppositories, compared with averages of 3,000 and 2,670 c.c. following the intravenous injection of mercupurin and salyrgan, respectively. Analogous data were reported by Fulton<sup>4</sup> and by Herrmann and Dechard.<sup>5</sup> The simultaneous administration of acid salts has been found to enhance greatly the activity of the mercurin suppositories.

Fulton,<sup>4</sup> Parkinson and Thomson,<sup>6</sup> Thomson,<sup>3</sup> and Herrmann and Dechard<sup>5</sup> all observed that the effect of the suppository was largely spent within twenty-four hours, but on the other hand, Peters<sup>7</sup> found that the diuresis occasionally lasted for forty-eight hours.

Rapidity of absorption has been demonstrated by Fulton,<sup>4</sup> Parkinson and Thomson,<sup>6</sup> and Engel<sup>2</sup> who observed active diuresis even in cases where the

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†This preparation, as well as mercupurin, is known in Europe as novurit.

suppositories were retained for only fifteen minutes to two hours. There is, of course, no evidence that absorption is always, or even usually, so rapid. It may be pointed out here that the majority of workers found the diuresis to be greater when a cleansing enema or an aperient preceded the administration of the drug.

Failures to obtain diuresis following the insertion of the suppositories have been comparatively few, although it is possible that all the failures may not have been reported. Thomson<sup>3</sup> obtained no diuresis in 12 of 200 instances in which suppositories were administered to 11 patients. Fulton<sup>4</sup> reported only 1 patient in a series of 25 who failed to respond to mercurin suppositories, although diuresis was obtained in this individual after parenterally administered mercurials. There were also 6 patients in his series who did not respond to either method of administration. It should be noted that, as with parenteral diuretics, patients whose edema was the result of cirrhosis of the liver usually were less responsive than those with edema of cardiac origin.

Engel<sup>8, 9</sup> has shown clinically that the excretion of mercury following the use of suppositories is quite irregular. This has been confirmed experimentally.<sup>10</sup>

In contrast to the marked unanimity of opinion concerning diuretic efficiency, there is considerable disagreement regarding the local toxicity of mercurial suppositories. Kisch,<sup>11</sup> Christian,<sup>12</sup> Kleeberg,<sup>13</sup> Herrmann and Dechard,<sup>5</sup> and Caughey<sup>14</sup> apparently observed neither subjective symptoms nor objective signs. Fulton,<sup>4</sup> Parkinson nad Thomson,<sup>6</sup> Thomson,<sup>3</sup> Engel,<sup>2</sup> and Wuhrmann<sup>15</sup> reported complaints of slight rectal burning, mild diarrhea, or desire for defecation, but considered these insignificant. Flexner<sup>16</sup> found that irritation occurred only in two cases of his series, and attributed this to the presence of hemorrhoids.

On the other hand, Natanson,<sup>1</sup> Dennig and Krause,<sup>17</sup> Budnitz,<sup>18</sup> and Peters<sup>7</sup> have attached more significance to the occasional occurrence of rectal burning, discomfort, tenesmus, and diarrhea. All of these workers have resorted to the addition of anesthetics or other agents to the suppositories in more or less successful attempts to relieve the subjective symptoms. Even Engel,<sup>8</sup> who considered the toxicity of the suppositories slight, found it desirable to use tincture of opium to relieve pain and discomfort. Furthermore, Engel<sup>2</sup> tested a suppository in which theophylline was combined with mercurin, but noted no change in the degree of irritation. Most clinicians agree that inflamed hemorrhoids, anal fissures, or proctitis are contraindications to the use of mercurial suppositories.

All the clinical reports cited concerning the local toxicity of mercurial suppositories have been based, so far as it is possible to determine, on the subjective symptoms of the patients to whom they were administered. Knowing that mercurin and salyrgan may cause local irritation and necrosis when administered intracutaneously or intramuscularly,<sup>19</sup> DeGraff, Cowett, and Batterman,<sup>20</sup> and at about the same time Crawford,<sup>21</sup> performed proctoscopic examinations on patients before and after suppositories were inserted. The results of

TABLE I  
SUMMARY OF LITERATURE ON MERCURIN SUPPOSITORIES

OBSERVER	YEAR	NO. OF CASES	TOTAL NO. OF SUPPOSITORIES FOR ALL PATIENTS	COMPARISON OF DIURETIC EFFICIENCY WITH THAT OF THE PARENTERAL MERCURIALS	LOCAL TOXICITY	AUXILIARY MEDICATION
Dennig and Krause	1935	22	ca. 34	Almost the same	In many patients burning, diarrhea, tenesmus	Anesthesin with suppository partially successful in preventing local irritation
Fulton	1936	25	90	Comparable to mercurial diuretics intravenously	Burning in a few patients	
Kisch	1936	3	4	Volume of diuresis about 90 per cent of that from mercurpurin	None	
Kleeberg	1936	Not reported	Not reported	Usually equal	None	
Peters	1936	28	Not reported	Slower in action but almost equal in volume	3 patients with burning, diarrhea, tenesmus	Anesthesin used to reduce toxicity
Engel	1937	10	Not reported	Slower in action but usually equal in volume	Not more than a sensation of heat and desire for defecation	Fincham of opium used to relieve pain
Herrmann and Dechard	1937	70	95	Volume of diuresis 80 and 85 per cent of that from mercurpurin and salyrgan, respectively (after enema)	None reported	
Parkinson and Thomson	1937	11	208	Volume of diuresis about 80 per cent of that from mercurial diuretics intravenously	4 patients, mild diarrhea 2 patients, slight discomfort 2 patients, considerable discomfort	
Caughy	1938	2	90	Not reported	None	
Flexner	1938	12	20	Equal	Irritation only in cases with previous rectal pathology	



this work, of which a summary is given in the first four rows of Table II, indicated that the subjective symptoms may be accompanied by visible rectal pathology. Out of a combined total of 34 patients who received the salyrgan suppository, 14 (41 per cent) showed rectal irritation, and 5 (15 per cent) definite ulcerations. No ulcerations were observed after the use of the mercurin suppository, and only 4 out of 31 patients (13 per cent) exhibited injection of the mucosa.

TABLE II

COMPARISON OF PROCTOSCOPIC FINDINGS AFTER RECTAL ADMINISTRATION OF MERCURIAL DIURETICS IN MAN

DRUG	NO. PATIENTS	NO. WITH SUBJECTIVE SYMPTOMS, BURNING, ETC.	PROCTOSCOPIC OBSERVATIONS		NO. PATIENTS WITH SYMPTOMS OF ANY KIND
			NO. WITH INJECTED MUCOSA	NO. WITH ULCERATIONS	
Mercurin (Crawford)	16	Not reported	4	0	4
Mercurin (DeGraff)	15	5	0	0	5
Salyrgan (Crawford)	16	Not reported	9	2	9
Salyrgan (DeGraff)	18	10	3	3	10
Modified salyrgan	36	4	2	0	4

From this review of the literature the following conclusions are reached: (1) The mercurin suppository is a useful diuretic. Its effectiveness is less than that of either salyrgan or mercurpurin administered parenterally. Its action is enhanced by premedication with ammonium chloride and by previously emptying the bowel by means of an aperient or enema. Insufficient published reports are available to warrant a statement concerning the diuretic efficiency of the salyrgan suppository. (2) Salyrgan is capable of producing distinct lesions in the rectal mucosa as well as pain, diarrhea, and tenesmus. Mercurin, while seldom producing demonstrable changes in the mucosa, occasionally gives rise to the same subjective symptoms. The presence of rectal pathology should be considered a contraindication to the use of these drugs in the form of suppositories.

In an effort to overcome the irritating properties of the salyrgan suppository, a number of modifications were tested experimentally in this laboratory. The results, which have been reported elsewhere,<sup>10</sup> indicated that a preparation composed of an equimolecular mixture of salyrgan sodium theophylline and salyrganic acid theophylline was the most advantageous. This drug, which will be called "modified salyrgan suppository," was found (in contrast to mercurin and salyrgan) to cause practically no rectal irritation in animals and to give rise to a twenty-four-hour urinary excretion of mercury which was equal to that after salyrgan.

Because these favorable results were noted experimentally, it was felt that clinical investigation of its local toxicity was warranted. Further, the diuretic activity of the modified salyrgan and of mercurin suppositories was studied in patients with congestive heart failure.

*Clinical Study of the Local Toxicity of the Modified Salyrgan Suppository.*—Thirty-six patients were chosen, regardless of clinical diagnosis, the only criterion being that they were in a satisfactory condition to allow proctoscopic

TABLE III

COMPARISON OF MERCURIN SUPPOSITORY (M. S.), MODIFIED SALYRGAN SUPPOSITORY (M. S. S.), MERCUPURIN (M.), AND SALYRGAN THEOPHYLLINE (S. T.) DIURETICS

CASE NO.	DIURETIC	DATE	AMM. CHLORIDE GR.	INITIAL WT.	WT. FIRST DAY	LOSS FIRST DAY	MAX. LOSS	DAYS REQUIRED FOR LOSS
1	M.—2 c.c.	4/ 7	6.0	129½	118	11½	11½	1
	M.—1 c.c.	4/21	6.0	109¼	104½*	5	5	1
	M. S.	5/13	6.0	118	116	2	2	1
	M.—2 c.c.	5/20	6.0	118	113½	4½	4½	1
2	M. S.	5/24	—	181	168½*	12½	12½	1
3	M.—2 c.c.	3/10	—	139	131*	8	9	2
	M. S.	3/23	—	135½	136½	0	0	—
	M.—2 c.c.	3/26	—	138	131½	6½	6½	1
4	M. S.	7/15	—	175	168½	6½	6½	1
	M. S.	7/19	—	170½	165½	5	5	1
	M. S.	7/24	6.0	173	165	8	8	1
	M.—2 c.c.	7/27	6.0	170	159	11	11	1
	M.—2 c.c.	7/30	6.0	163	154	9	9	1
5	M.—2 c.c.	6/19	—	128½	128	0	0	—
	M. S.	6/26	3.0	129	129	0	0	—
	M.—2 c.c.	6/29	6.0	130	126½	3½	3½	1
	M.—2 c.c.	7/27	—	113¾	110¼	5½	6½	2
6	M. S.	5/19	—	111	113½	0	0	—
	M.—2 c.c.	5/23	—	113	109	4	4	1
7	M. S.	5/19	—	118	115	3	11	3
	M. S.	6/ 5	—	113	107½	5½	8½	2
	M. S.	6/20	Theocin, 2 Gm. per day	111	101*	10	14½	2
8	M.—2 c.c.	4/28	6.0	211½	204¼	7¼	8	2
	M.—2 c.c.	5/ 4	6.0	200¼	198½	1¾	1¾	1
	M.—2 c.c.	5/ 9	6.0	194¾	190½	4¼	5¾	2
	M. S.	5/20	6.0	186½	185½	1	1	1
9	M.—2 c.c.	5/17	—	194½	188	6½	6½	1
	M. S.	5/23	—	193	177	16	16	1
	M.—2 c.c.	6/ 5	—	182	175	7	7	1
	M. S.	6/10	—	182	175½	6½	6	2
	M. S.	6/18	—	183	180½	2½	2½	1
	M. S.	6/29	—	181	174½	6½	6½	1
	M. S.	7/ 6	—	186	181	5	5	1
	M.—2 c.c.	7/15	—	194	188	6	6	1
10	M.—2 c.c.	5/ 9	4.0	165½	157	8½	8½	1
	M. S.	5/26	6.0	160	131½	8½	8½	1
	M.—2 c.c.	6/ 2	6.0	157½	145½*	12	12	1
	M.—2 c.c.	6/ 8	6.0	149½	145½	4	4	1
11	M. S. S.	9/19	6.0	149¼	148	1¼	1¼	1
	M.—2 c.c.	9/28	6.0	148	139*	9	10	2
12	M. S. S.	9/ 5	—	146	145½	½	½	1
	M.—2 c.c.	9/ 8	—	146½	142	4½	8½	2
	M. S. S.	9/14	3.0	138	132*	6	6½	2
13	M. S. S.	11/26	—	204	200	4	4	1
	M.—2 c.c.	11/29	—	199	185½	13½	14½	2

\*Weight at which patient was edema free.

TABLE III—CONT'D

CASE NO.	DIURETIC	DATE	AMM. CHLORIDE GM.	INITIAL WT.	WT. FIRST DAY	LOSS FIRST DAY	MAX. LOSS	DAYS REQUIRED FOR LOSS
14	M. S. S.	9/30	6.0	111½	110	1½	4½	2
	M.—2 c.c.	10/12	6.0	107	94	13	?	?
	M.—2 c.c.	10/13	6.0	94	90	4	4	1
15	M.—2 c.c.	8/ 5	3.0	143	132	11	11	1
	M. S. S.	8/17	3.0	138	135	3	3	1
	M.—2 c.c.	8/22	3.0	139½	131½	8	8	1
	M. S. S.	8/29	3.0	139½	138½	1	3½	2
	M.—2 c.c.	9/ 2	3.0	137½	131*	6½	6½	1
	M. S.	9/13	3.0	139	138	1	2	2
16	M. S. S.	12/14	3.0	134	130	4	8	2
	M.—2 c.c.	12/17	3.0	123	113	10	10	1
17	M.—2 c.c.	12/13	3.0	163½	156½	7	8	2
	M. S. S.	12/19	3.0	157½	157	½	½	1
	M. S. S.	12/21	3.0	158½	160	-	0	-
	S. T.—2 c.c.	12/22	3.0	160	156	4	4	1
	M. S. S.	12/26	3.0	158	154	4	6	2
18	M.—2 c.c.	12/12	3.0	128	125	3	3	1
	M.—2 c.c.	12/14	3.0	128	124	4	4	1
	M.—2 c.c.	12/17	3.0	127½	122½	5	5	1
	M.—2 c.c.	1/11	3.0	145	137	8	8	1
	M. S. S.	1/16	3.0	141½	143	-	0	-
	M. S. S.	1/18	3.0	145	143	2	2	1
	S. T.—2 c.c.	1/21	3.0	145½	140½	4	4	1
	M. S. S.	1/23	3.0	142	142½	-	0	1
	M.—2 c.c.	1/24	3.0	142½	134½	8	10½	2
	M. S.	1/28	3.0	139	139½	-	0	1
	M.—2 c.c.	1/29	3.0	139½	131	8½	8½	1
	M. S. S.	2/ 3	3.0	137½	137	½	½	1
	S. T.—2 c.c.	2/ 4	3.0	137	130½	6½	7½	2
	M.—2 c.c.	2/ 8	3.0	134	130½	3½	3½	1
19	M. S. S.	3/ 1	3.0	163	157¾	5¼	6	2
	M. S. S.	3/ 8	3.0	160	156½	3½	5½	2
20	M. S. S.	3/22	6.0	185½	184	1½	1½	1
	M. S.	3/27	6.0	185	178	7	7	1
	M. S. S.	3/31	6.0	181	177	4	4	1
	M. S.	4/ 4	6.0	178½	172	6½	6½	1
21	M. S. S.	3/31	3.0	147¾	146	1¾	1¾	1

TABLE IV

CASE NO.	DIURETIC	DATE	AMM. CHLORIDE GM.	AVERAGE URINARY OUTPUT IN C.C. BEFORE DIURETIC	URINARY OUTPUT IN C.C. AFTER DIURETIC	DAYS REQUIRED FOR LOSS
22	M. S. S.	11/17	3.0	958	2,683	1
	M. S. S.	11/20	3.0	1,283	2,775	1
	M. S. S.	11/23	3.0	1,256	2,425	2
	M.—2 c.c.	11/26	3.0	965	3,540	1
	M. S. S.	11/27	3.0	686	1,920	1
	S. T.—2 c.c.	12/ 2	3.0	611	2,160	1
	M. S.	12/ 6	3.0	794	1,010	-
	S. T.—2 c.c.	12/ 9	3.0	660	2,160	1
	M.—2 c.c.	12/13	3.0	868	2,330	1
	M.—2 c.c.	12/16	3.0	795	1,900	1
	M.—2 c.c.	12/21	3.0	745	2,950	1

examination. A history with reference to previous rectal disease was carefully recorded, and the presence of hemorrhoids, fissures, and other lesions was noted. Proctoscopic examinations\* were made shortly after a cleansing enema and before the suppositories were inserted. The patients were instructed to avoid defecation, if possible, for twenty-four hours. Proctoscopic examinations were repeated in each case twenty-four hours after the administration of the mercurial. Routine urine examinations for albumin and formed elements were in every instance essentially negative.

Of the 36 patients studied, 4 complained of a burning sensation following the insertion of the suppository. Upon proctoscopy, one of this group of 4, and one other who had had no subjective symptoms, exhibited definite injection of the rectal mucosa. No instances of ulceration were observed. Examination before administration of the drug revealed internal hemorrhoids in each of these 5 patients. The results, however, cannot be assigned entirely to the initial pathology, for 12 other cases with hemorrhoids, 2 of which were ulcerated and one case with anal fissure, were entirely free from symptoms, and the rectal mucosa appeared unchanged. While it is not advisable to use any mercurial suppository in the presence of ulcerated hemorrhoids and anal fissures, the absence of toxicity in the foregoing cases emphasized the low degree of irritating properties of the modified salyrgan suppository.

The results are summarized in the last row of Table II and are thus compared with those obtained under similar condition of study for salyrgan and mercurin. It is evident that the modified salyrgan suppository is distinctly superior to the original salyrgan suppository, and is at least no more irritating than the mercurin suppository.

*Clinical Study of Diuretic Potency of Modified Salyrgan Mercurin Suppositories.*—The diuretic effectiveness of mercurin and modified salyrgan suppositories has been investigated in 22 patients with congestive heart failure. The mercurin suppository contains 200 mg. of mercury, and the modified salyrgan suppository,† 158 mg. of mercury. The suppositories were administered only after the patients had had a preliminary period of bed rest and the weight had become constant. In those receiving digitalis, the diuretic was not given until complete digitalization was obtained and the weight remained constant on a maintenance dosage. In following the diuretic response, it was found to be more accurate to follow the daily weight curve, as this fluctuated far less than the urinary output. It also was a good guide in ascertaining the amount of fluid available in the body for diuresis. It has previously been emphasized<sup>22</sup> that the range between the weight before and after diuresis, and the level obtained when the patient is edema-free, is the best available indicator for the effectiveness of a diuretic. Patients were weighed at the same time each day on a scale accurate to 2 ounces. The daily fluid intake was limited to 1,200 c.c., and the salt intake was approximately 5 Gm. Ammonium chloride was given to 16 patients in doses of 3 to 6 Gm. daily.

\*We are indebted to Dr. Max P. Cowett, proctologist to the Third Medical Division of Bellevue Hospital, for his interest and kindness in supervising many of these examinations.

†The mercurin suppository was the same as that in general use and was supplied by Campbell Products, Inc. The modified salyrgan suppository was supplied by the Winthrop Chemical Co. under the research number ST 3815.

Three hours after a cleansing enema, the suppository was inserted and the patient was advised to retain it as long as possible. In none of the cases was the suppository expelled in less than six hours, and in most of them it was retained almost twenty-four hours.

The protocols are presented in Tables III and IV. The mercurin suppository was administered 23 times to 14 patients, and the modified salyrgan suppository was given 24 times to 12 patients, 4 patients receiving both preparations. Seventeen patients also received mercurial diuretics intravenously. In 8 of these the suppository was given prior to the parenteral therapy.

A loss of at least 3 pounds in body weight within forty-eight hours of the administration of the diuretic was considered a significant response. In one patient who was too ill to be weighed and for whom the daily urinary output had to be relied upon to determine diuresis, a urinary volume of twice the basal level was considered significant.

A summary of the responses to the trials with the mercurial diuretics is presented in Table V. The percentages of significant diuretic responses with both types of suppository are definitely low compared with that of the parenterally administered diuretic. These results would indicate that while good diuresis may follow the administration of a mercurial diuretic in the form of a suppository, it could be depended upon only in 60 per cent of the cases.

TABLE V

SUMMARY OF TRIALS WITH MERCURIAL DIURETICS SHOWING TOTAL SIGNIFICANT RESPONSES

Salyrgan theophylline was employed intravenously 5 times during the study, but because of the small number of cases, tabulation of the results is omitted.

DIURETIC	TRIALS	DIURETIC RESPONSE	FAILURES	PER CENT SUCCESSES
Mercurin suppository	23	14	9	60.8
Modified salyrgan suppository	24	14	10	58.3
Mercurpurin, intravenously	41	39	2	95.1

In the majority of cases the diuresis was complete within twenty-four hours. However, in eight instances following administration of either suppository, a further loss of 2 pounds or more within the next twenty-four hours, and in one instance a loss of 8 pounds during the next forty-eight hours, were noted. Following intravenous administration, there were two instances of significant weight loss during the second twenty-four-hour period.

One patient responded to suppositories with significant diuresis after ammonium chloride therapy had been started, whereas earlier trials had been unsuccessful. While we are in agreement with others that acid salts enhance the action of mercurial diuretics, several failures to respond to mercurial suppositories occurred during the administration of ammonium chloride.

*Evidences of Renal Irritation.*—Two patients showed changes in the urine during these investigations. Case 4, a 74-year-old man with arteriosclerotic and hypertensive heart disease, showed increasing albuminuria after the third mercurin suppository and, following the first intravenous injection of mercurpurin three days later, developed marked albuminuria, innumerable hyaline

and granular casts, and white blood cells. The patient died suddenly three weeks later. At autopsy, the kidneys were normal, grossly, but microscopically the collecting tubules exhibited an occasional calcium deposit in their walls. Case 5, a 38-year-old man with active rheumatic heart disease, showed similar urinary changes following an intravenous injection of mercurpurin, given three days after a mercurin suppository had proved ineffective. Cessation of the use of all mercurial diuretics resulted in a return to normal within three weeks, after which time mercurpurin was repeated with resulting good diuresis and absence of evidences of renal irritation.

#### DISCUSSION

The mercurin and modified salyrgan suppositories have been shown to produce minimal demonstrable changes in the rectal mucosa, although they may at times produce local discomfort and burning.

The mercurial suppository is an efficient diuretic, but it can be depended upon only 60 per cent of the times it is administered. The results with the modified salyrgan suppository are less striking than those produced by the mercurin suppository, but it must be remembered that the former contains 158 mg. of mercury compared to 200 mg. in the latter.

The problem arises as to what further therapy would be advisable should the suppository fail to promote diuresis where this is imperative for the welfare of the patient. If diuresis does not occur, it is interesting to speculate that possibly that mercury absorbed from the rectum continues to accumulate in the kidney in amounts that normally would have been excreted. Therefore, if a parenteral diuretic is subsequently given, additional mercury reaches the kidney and may be sufficient to produce tubular damage. The two patients presenting evidence of renal irritation, when an intravenous mercurial was given three days after the suppository, may represent examples of such an occurrence. Therefore, it is to be emphasized that, if the diuresis is not satisfactory after the rectal administration of a mercurial diuretic, the parenteral route should be resorted to only after an appreciable time interval has elapsed to allow the body to care adequately for the previously absorbed mercury.

#### SUMMARY

1. The mercurin and modified salyrgan suppositories produce only minimal changes in the rectal mucosa, but they may occasionally cause local discomfort and burning.

2. Mercurial diuretic suppositories may produce a definite and clinically effective diuresis, but their dependability is only 60 per cent.

3. The possible danger of administering a mercurial diuretic intravenously soon after a suppository is discussed.

We are very grateful to Dr. Arthur C. DeGraff under whose guidance these investigations were conducted.

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# THE EXCRETION OF SQUASH SEED GLOBULIN AND BLOOD PROTEINS IN THE URINE AFTER INTRAVENOUS INJECTION OF CRYSTALLIZED SQUASH SEED GLOBULIN\*

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THE fact that the parenteral injection of foreign proteins into animals causes proteinuria has been recognized for some time. Since Berzelius' observation of this phenomenon, a number of studies of the effect of the injection of foreign protein have been reported in the literature. Inasmuch as the literature on this subject has been recently reviewed by Briggs,<sup>1</sup> only those papers more specifically related to the present discussion will be cited here. In summary, it may be stated that most of these previous investigations have been carried out with complex mixtures of antigenic proteins, such as egg white and blood sera. The excretion of proteins was tested for, in almost all cases, by means of chemical tests which are not sufficiently delicate to detect very small traces of protein, and are not, except in rare instances,<sup>†</sup> capable of identifying the protein. It seemed desirable, therefore, to study the elimination of a pure protein and to investigate at the same time the excretion of blood proteins following the injection of the foreign protein. Two such studies have been carried out in this laboratory, one using crystallized ovalbumin, the other using crystallized squash seed globulin for injection. The two studies were carried out for the most part simultaneously and, as far as possible, by the use of similar techniques. The excretion of proteins in the urine of injected dogs was followed by testing for the injected protein and for dog serum albumin and pseudoglobulin by means of the precipitin reaction.

The results of the investigation using crystallized ovalbumin have already been reported by Briggs.<sup>1</sup> The results of experiments with crystallized squash seed globulin are reported in this paper. This protein had not previously been used in this type of study. Two somewhat similar proteins, edestin and excelsin, were used by Mendel and Rockwood,<sup>2</sup> who reported that they were not excreted in unchanged form. The tests used by these investigators, however, were not capable of detecting very small traces of protein.

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†In some cases the protein used for injection allowed more specific deductions to be made. Thus Pavy,<sup>2a</sup> after the injection of isinglass, detected both gelatin and coagulable protein in the urine. Parisot and his co-workers<sup>2b</sup> were able to show the excretion of globin and blood proteins following the injection of globin.



## PREPARATION OF MATERIAL

The proteins used were prepared in as pure a form as possible. Crystallized squash seed globulin was prepared according to Osborne's method,<sup>4</sup> and purified by four recrystallizations. The blood proteins were prepared by Welker's method.<sup>5</sup> All antisera for these proteins were obtained by intramuscular injection of rabbits with the individual proteins adsorbed on aluminum hydroxide, according to the method of Hektoen and Welker.<sup>6</sup> Each antiserum had a titer of at least 1:100,000, and was specific for the antigen used in its preparation.

## PRECIPITIN TESTS

All precipitin tests were made by the contact layer method. A drop of the solution to be tested in progressive dilutions was layered over a drop of antiserum, and the highest dilution of the substance which would give a white band precipitate at the point of contact after standing for one hour at room temperature was determined.

## IMMEDIATE EFFECT OF A FOREIGN PROTEIN ON THE KIDNEY

In order to determine the immediate effect of a foreign protein on the kidney of the dog, three medium-sized dogs were injected with crystallized globulin, and urine specimens were collected for an hour. This was accomplished by inserting a No. 8 hard rubber catheter into the bladder of a male dog, and after a preliminary period of ten to twenty minutes, introducing 10 c.c. of a 1 per cent solution of crystallized squash seed globulin in 6 to 7 per cent sodium chloride solution into the lesser saphenous vein. The urine excreted over short time intervals was collected and tested by the precipitin reaction for the presence of crystallized squash seed globulin, dog serum pseudoglobulin, and dog serum albumin. In all three experiments the urine before the injection of the foreign protein gave negative tests for both dog blood proteins. The results of these experiments are summarized in Table I.

TABLE I

TIME OF INITIAL APPEARANCE OF PROTEINS IN THE URINE FOLLOWING THE INJECTION OF CRYSTALLIZED SQUASH SEED GLOBULIN

DOG	WEIGHT IN KG.	MINUTES AFTER INJECTION OF SQUASH SEED GLOBULIN BEFORE APPEARANCE OF		
		CRYSTALLIZED SQUASH SEED GLOBULIN	SERUM PSEUDOGLOBULIN	SERUM ALBUMIN
31	8.2	7	7	14
32	6.4	10	10	15
33	6.8	5	5	5

*Effect of Repeated Injections of a Pure Foreign Protein on the Elimination of the Foreign Protein and the Blood Proteins.*—Male dogs, weighing 6 to 14 kg., were used as experimental animals. During the period of injection the dogs were kept in metabolism cages on a complete diet, and the urine was collected under toluene. Each series of injections was preceded by a control period varying from ten to eighty days. During the control period the animals were

kept under experimental conditions, and occasional samples of urine were collected and examined for the presence of dog serum pseudoglobulin and dog serum albumin.

One per cent solutions of crystallized squash seed globulin dissolved in 6 to 7 per cent sodium chloride solution were injected into the leg veins. The injections were repeated within two to six days, so that the physiologic action would take place in an immune, rather than a sensitive, animal. After the completion of the series of injections, the dogs were kept in stock pens, or cages, and returned to the metabolism cages occasionally for periods of observation. The urine specimens were filtered and tested by the precipitin reaction for the presence of crystallized squash seed globulin, dog serum pseudoglobulin, and dog serum albumin.

Ten adult male dogs and one female puppy were given 4 to 35 injections of crystallized squash seed globulin. The duration of the injection period was determined by the effect of the solution injected on the veins of the dog's legs. In some animals the solution caused rapid occlusion of the vessels; in others this action appeared more slowly. The animals may be divided into the following groups:

Group 1.—Dogs 1, 2, 3, 5, and 10 received from 64 to 152 c.c. of 1 per cent squash seed globulin in from 6 to 13 doses, extending over periods of from thirteen to ninety-eight days. Dog 4 received 528 c.c. of this foreign protein solution in 35 injections during a period of one hundred and five days.

Group 2.—Dogs 7, 8, 9, and 11 received from 10 to 72 c.c. of the foreign protein solution in from 10 to 20 doses, extending over periods of from fifty-seven to one hundred days.

Group 3.—Dog 6 received 202 c.c. of 1 per cent squash seed globulin solution in 4 injections over a period of ten days.

TABLE II  
REPEATED INJECTION OF CRYSTALLIZED SQUASH SEED GLOBULIN

DOG	WEIGHT IN KG.	NO. OF INJECTIONS	AMOUNT INJECTED IN GM.	DURATION OF INJECTION PERIOD IN DAYS	DURATION OF OBSERVATION PERIOD AFTER LAST INJECTION IN DAYS
1	11.0	8	0.79	24	1,555
2	7.7	6	1.52	21	2
3	11.3	7	1.44	13	7
4	11.3	35	5.28	105	33
5	9.1	6	0.64	14	7
10	10.0	13	1.05	98	52
7	13.6	10	0.38	57	336
8	10.9	20	0.72	113	Died
9	5.6	5	0.10	21	57
11	9.1	11	0.51	85	Died
6	9.3	4	2.02	10	

Table II shows the amount of squash seed globulin injected into each animal, the duration of the injection period, and the length of the period of observation following the last injection.

## EXCRETION OF SQUASH SEED GLOBULIN

In the case of 5 of the 6 dogs of group 1, analysis of the urine excreted during the injection period showed that about 35 per cent of the urine samples contained squash seed globulin. In the case of Dog 10, however, only 3 of 57 specimens gave a positive test for the protein injected. With the dogs of group 2, which received smaller doses of foreign protein, a smaller fraction (about 15 per cent) of the urine excreted during the injection period gave positive reactions for the protein injected. One dog of this group, Dog 11, showed little tendency to excrete squash seed globulin, which appeared in but 3 of 48 samples. These results are summarized in Table III.

TABLE III  
EXCRETION OF SQUASH SEED GLOBULIN

DOG	NUMBER OF TESTS MADE*	POSITIVE TESTS WITH DIFFERENT DILUTIONS OF URINE					TOTAL NUMBER OF POSITIVE TESTS	PERCENTAGE OF TESTS POSITIVE
		UNDILUTED	1:10	1:100	1:1,000	1:10,000		
1	54	10	4	4	1	2	21	39
2	26	6	2	1	0	0	9	35
3	17	4	0	1	0	0	5	29
4	104	36	1	1	0	0	38	35
5	18	7	0	0	0	0	7	39
10	57	0	0	1	0	2	3	5
7	53	9	0	0	0	0	9	17
8	97	12	2	0	0	0	14	14
9	18	3	1	0	0	0	4	22
11	48	0	0	0	0	3	3	6
6	8	3	2	0	0	0	5	63

\*These figures include urine excreted for seven days following the last injection, except in the following cases: Dogs 6 and 9 died following the last injection, and only one urine sample was obtained after the final injection. Dog 2 died two days after the final injection.

In the animals of group 1 the excretion of squash seed globulin decreases after the first 3 or 4 injections. This tendency is shown in Table IV. Opie<sup>7</sup>

TABLE IV  
PER CENT OF URINE SPECIMENS GIVING POSITIVE REACTIONS FOR SQUASH SEED GLOBULIN AT DIFFERENT STAGES OF THE INJECTION PERIOD

DOG	DURING THE FIRST PART OF INJECTION PERIOD	DURING THE LAST PART OF INJECTION PERIOD
1	50	27
3	44	13
4	55	27
5	55	22

has reported similar findings with ovalbumin. He expressed the belief that as immunization proceeds the injected animal acquires an increased ability to get rid of the injected protein. Some of Briggs' findings<sup>1</sup> are in accord with Opie's opinion.

After the final injection of crystallized squash seed globulin, the elimination of this protein practically ceases after two to four days. Occasional samples, however, show the presence of the foreign protein for a period of eight to twenty-one days after the final injection. Dog 1 was observed, one

TABLE V  
EXCRETION OF BLOOD PROTEINS

OF BLOOD PROTEINS

DOG	BEFORE INJECTION			AFTER INJECTION						
	NO. OF TESTS MADE	POSITIVE TESTS		NO. OF TESTS MADE	POSITIVE TESTS		NO. OF POSITIVE TESTS IN HIGHER DILUTIONS			PER CENT OF POSITIVE TESTS IN HIGHER DILUTIONS
		NO.	%		NO.	%	1:10	1:100	1:1,000	
<i>Pseudoglobulin</i>										
1	5	0	0	131	124	95	69	5	0	56
2	6	3	50	25	25	100	15	0	0	60
3	5	5	100	17	17	100	9	2	0	65
4	6	1	17	140	103	74	8	1	0	6
5	6	3	50	19	15	79	0	0	0	0
10	20	15	80	77	69	90	18	0	0	23
7	26	9	35	58	53	91	10	0	0	17
8	54	17	32	102	88	85	9	0	0	9
9	13	10	77	18	8	44	1	2	0	17
11	14	5	36	74	58	78	11	0	0	15
6	15	14	93	8	7	88	3	1	0	50
<i>Albumin</i>										
1	5	0	0	131	124	95	41	2	1	34
2	6	2	33	25	25	100	17	0	0	67
3	5	5	100	17	17	100	10	2	0	71
4	6	4	67	140	131	94	16	1	0	12
5	6	6	100	19	19	100	1	2	0	5
10	20	20	100	77	73	95	18	0	0	26
7	26	5	19	58	32	55	6	0	0	10
8	54	15	28	102	63	62	5	2	0	7
9	13	5	38	18	8	44	3	1	0	22
11	14	5	36	74	69	93	16	0	0	22
6	15	15	100	8	8	100	5	0	1	75

year after the final injection, to resume the excretion of small traces of squash seed globulin in several urine samples over a period of twenty-three days (see Table VI).

The amount of squash seed globulin excreted represents only a small part of that injected. The major portion must be either stored in the tissues, metabolized in the body, or excreted in a form which does not react with the specific antiserum. Such an immunologically altered form might result from the combination of the foreign protein with blood proteins. Welker, Andrews, and Thomas<sup>8</sup> have suggested that albuminuria is a result of a protective mechanism in which toxic substances are combined with blood proteins with the formation of a foreign protein which is excreted. It was not possible in this investigation to show that crystallized squash seed globulin formed such combinations.

Hunter<sup>9</sup> has expressed the opinion that the nonexcreted fraction of parenterally injected foreign proteins is digested by proteolytic enzymes elaborated by leucocytes. This hypothesis is based on his observation that during the immunization of rabbits with ox serum and ox serum proteins, each injection was followed by a leucocytosis which increased with each injection.

#### EXCRETION OF BLOOD PROTEINS

Before injection the urine of most of the dogs gave occasional tests for one or both of the blood proteins. Such positive tests were obtained only on

the undiluted urine, never, except with Dog 6, with urine that had been diluted with 9 or more parts of saline. Immediately after the injection of squash seed globulin, the excretion of the blood proteins was increased. This increased excretion can be noted in the greatly increased frequency with which the urine gives positive tests for these proteins, and by the fact that a number of samples after injection give positive tests when diluted with 9 parts of saline; occasional samples give positive tests when diluted with 99 or more parts of saline. The increase in the excretion of blood proteins was observed in all the dogs tested. The findings are listed in Table V. This increase in the excretion of blood proteins continues after the final injection of squash seed globulin has been given and the foreign protein no longer appears in the urine.

The excretion of foreign protein was always accompanied by the elimination of one or both blood proteins. Two different dogs on one occasion excreted hemoglobin as well as the two plasma proteins. These urines did not contain red blood cells nor did they hemolyze dog erythrocytes. Squash seed globulin was present in one of these hemoglobin-containing urines but not in the other.

The excretion of blood proteins continues long after the urine has ceased to contain squash seed globulin. The excretion of these proteins cannot be correlated with any known factor. Samples usually contained both blood proteins in equal amounts; at times the amount of pseudoglobulin was greater than that of albumin, at other times it was less. Some urine specimens contained only one of the serum proteins, and occasional samples were protein free. The ratio in which these proteins were excreted was not related either to the difference in their molecular size nor to the difference in their concentration in the blood. The process, therefore, cannot be considered as one of simple filtration.

Table VI gives the results of the urine analyses of Dog 1. The results are, for the most part, typical of those obtained with the animals of group 1.

The amounts of protein found in these urines were too small to be detected by the usual chemical tests and would not be considered clinically as pathologic. On the occasions when hemoglobin was excreted, the condition was only transitory and cleared up within a few hours. The histologic examination of sections of liver, spleen, and kidney showed no changes which could be attributed to the experimental procedure. These results are similar to those obtained by Briggs<sup>1</sup> in her study with ovalbumin. Brull<sup>10</sup> has found that dogs can excrete egg white for weeks without presenting a subsequent tendency to true proteinuria.

#### SUMMARY

The injection of crystallized squash seed globulin into the blood of medium-sized dogs was followed by its appearance in the urine within five to ten minutes. It continues to be excreted for a period of twenty-four to seventy-two hours.

TABLE VI

EXCRETION OF SQUASH SEED GLOBULIN AND BLOOD PROTEINS FOLLOWING INTRAVENOUS INJECTION OF CRYSTALLIZED SQUASH SEED GLOBULIN (DOG 1)

TIME AFTER INITIAL INJECTION IN HOURS	AMOUNT OF 1% CRYST. SQUASH SEED GLOBULIN INJECTED IN C.C.	PRECIPITIN REACTIONS OF URINE WITH ANTISERUM FOR		
		CRYST. SQUASH SEED GLOBULIN	SERUM PSEUDO- GLOBULIN	SERUM ALBUMIN
Control		0	0	0
5	5	4	2	2
22		5	1	1
24		3	1	1
45		1	1	1
72		0	1	1
96		0	0	1
124		0	1	1
147		0	1	0
171	5			
173		0	2	2
189		2	2	1
193		5	3	1
216		2	2	0
241		0	2	1
262		1	2	2
286		0	0	1
288	10			
291		1	1	1
294		3	3	1
309		2	1	2
314		0	3	1
334		3	2	1
339		1	2	1
340		0	2	2
362	15	0	2	2
364		1	1	1
381		1	0	1
405		0	2	1
429		0	1	1
436		0	2	1
457	9			
459		0	2	3
460		1	2	2
476		1	3	4
478	17			
480		1	1	1
481		0	2	2
500		0	2	2
523		0	2	1
526	5			
527		0	2	1
548		0	1	2
561		3	2	1
595		0	1	1
598	13			
602		0	1	1
620		0	2	1
626		1	2	2
644		1	2	2
668		0	1	1
692		0	2	3
706		0	2	1
763		0	2	1
768		0	1	1
812		0	2	1
836		0	2	2
860		0	2	2
884		3	2	2
911		0	2	2
958		0	2	2
1,006		1	1	2

TABLE VI—CONT'D

TIME AFTER INITIAL INJECTION IN DAYS	AMOUNT OF 1% CRYST. SQUASH SEED GLOBULIN INJECTED IN C.C.	PRECIPITIN REACTIONS OF URINE WITH ANTISERUM FOR		
		CRYST. SQUASH SEED GLOBULIN	SERUM PSEUDO- GLOBULIN	SERUM ALBUMIN
43		0	1	2
45		2	0	1
46		0	0	0
49		0	0	0
50		0	0	1
51		0	1	2
52		0	1	2
53		0	1	1
220		0	2	2
221		0	1	1
222		0	1	1
385		0	1	1
388		0	1	2
390		0	2	1
391		2	2	2
391		1	2	1
394		0	2	1
395		0	2	2
396		0	2	2
397		0	2	1
398		1	1	1
399		0	2	1
400		0	0	0
401		0	2	1
403		0	1	1
404		0	2	1
405		1	2	1
407		1	2	1
408		1	2	2
409		0	2	2
410		1	2	1
412		1	2	1
414		1	1	1
415		0	2	1
416		0	1	1
425		0	2	1
426		0	2	1
428		0	1	1
429		0	1	1
431		0	1	1
432		0	2	1
434		0	1	1
435		1	1	1
436		0	1	1
437		0	1	1
438		0	1	1
774		0	2	1
775		0	1	1
776		0	2	1
781		0	1	1
782		0	2	2
785		0	2	2
1,172		0	2	2
1,173		0	2	1
1,174		0	2	2
1,574		0	2	2
1,575		0	2	2
1,576		0	1	1
1,577		0	3	0
1,578		0	2	2

0 = No reaction.

1 = Reaction in undiluted urine.

2 = Reaction in all specimens through the 1:10 dilution.

3 = Reaction in all specimens through the 1:100 dilution.

4 = Reaction in all specimens through the 1:1,000 dilution.

5 = Reaction in all specimens through the 1:10,000 dilution.

Following repeated injections, the amount of squash seed globulin excreted tends to decrease. Some foreign protein is excreted at irregular intervals after the last injection.

The amount of squash seed globulin excreted represents only a small portion of that injected. The larger portion could not be accounted for.

The appearance of squash seed globulin in the urine was always accompanied by either serum albumin or serum pseudoglobulin, or both. Repeated injection of squash seed globulin did not cause an increase in the amount of serum proteins excreted.

The injection of crystallized squash seed globulin into medium-sized dogs caused neither immediate kidney damage nor had a cumulative effect on the kidney.

I wish to express my appreciation to Dr. William H. Welker for his advice in the conduct of this investigation and to Dr. George Milles for his kindness and cooperation in examining the histologic material.

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## THE FASTING EXERCISE BLOOD SUGAR CURVE: A GUIDE FOR THERAPY IN DIABETES MELLITUS\*

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INCREASING experience with protamine zinc insulin has brought into sharp relief a difference in its effectiveness in persons with mild and severe diabetes. The degree of severity seems to be a basic factor in this form of therapy, and it appears that by measuring it, a guide for the efficient use of protamine zinc insulin might be established.

Efforts toward determining the type of diabetes were made long before the introduction of the longer lasting insulins. Petrén<sup>1</sup> early observed that in some diabetic persons the blood sugar curve tended to drop toward the afternoon, irrespective of meals. Schmidt<sup>2</sup> classified diabetic persons as insulin sensitive if they were asthenic, and as insulin insensitive if they were sthenic. Hinsworth<sup>3</sup> and MacBryde<sup>4</sup> developed insulin sensitivity tests designed to differentiate good from poor responses to standard insulin. Klatskin,<sup>5</sup> however, has recently pointed out that the division of diabetic persons into relatively insulin sensitive and resistant groups is an artificial one, while Joslin feels that it is not so simple to classify persons with diabetes.

Of particular interest is a practical method proposed by Seyderhelm and Oesterich<sup>6</sup> for predetermining the need for standard insulin. Diabetic patients were given a small carbohydrate meal the evening before, after the first blood specimen the following morning, and again at noon. Blood specimens were taken while fasting in the morning, and before the noon and evening meals, and the values were plotted as the pattern for the particular patient. Those showing a distinct reduction in the noon value were considered benign and did not require insulin. Where the curve followed an ascending course, the diabetes was severe and required insulin.

More recently, Greene<sup>7</sup> has conducted fasting blood sugar studies which offer a rational basis for grading the severity of diabetes and determining the potential response to protamine zinc insulin. Three types of blood sugar patterns were observed: (1) those in which all readings were within normal limits; (2) those in which the first reading was above normal, with a drop in subsequent determinations on continued fasting; and (3) those in which the first reading was above normal, with a rise in subsequent determinations on prolonged starvation. In group 2 it was evident that endogenous insulin was available, while in group 3 it was lacking. The former, persons with mild diabetes, would do well on Allen's starvation regime, while the latter, persons with more severe diabetes, would require insulin.

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From these observations of Greene and the studies of Seyderhelm and Oesterich, it was believed that a method might be devised whereby the severity of diabetes could be gauged and the response to treatment with protamine zinc insulin could be predicted. Since Greene's classification of diabetic persons studied during starvation appears fully as adequate as that of Seyderhelm and Oesterich, whose patients were given small carbohydrate meals, it was decided to develop patterns during starvation. However, a shortening of the time allotted for the study of each patient seemed desirable both from the practical standpoint and for the protection of the severe diabetic person from serious acidosis. Observation soon disclosed that patients could comfortably tolerate a six-hour period of study without undue risk and with no added harm if they were allowed to exercise or to continue at their regular work. The addition of this latter factor, exercise, served to emphasize the distinction between the mild and the severe forms of the disease, since it has been shown by Marble and Smith<sup>8</sup> that exercise increases the consumption of sugar in normal persons and in patients with mild diabetes, and decreases it in persons with severe diabetes.

TABLE I

NO.	PATIENT	AGE	BLOOD SUGAR			THERAPY
			INITIAL	AT 3 HR.	AT 6 HR.	
1	J. B. C.	60	280	190	184	Diet
2	M. S.	56	142	111	86	Diet
3	L. B.	41	80	71	57	Diet
4	Mrs. M. B.	51	125	115	87	Diet
5	N. G.	47	133	115	106	Diet
6	Mrs. P. D.	66	143	121	120	Diet
7	Mrs. W. S.	59	80	77	76	Diet
8	O. M.	61	100	108	100	Diet
9	Mrs. B. T.	48	180	151	133	Diet
10	Mrs. E. E.	59	220	160	166	Diet
11	Mrs. L. Z.	57	210	186	133	Diet
12	Mrs. M. Z.	49	170	142	133	Diet
13	Mrs. R. B.	50	144	125	95	Diet
14	Mrs. M. P.	66	166	153	129	Diet
15	V. A.	49	105	105	87	Diet

TABLE II

NO.	PATIENT	AGE	BLOOD SUGAR			THERAPY
			INITIAL	AT 3 HR.	AT 6 HR.	
49	J. M.	30	192	152	133	25 PZI
50	Mrs. S. G.	57	88	69	69	10 Standard
						20 PZI

As for the usefulness of the procedure in predicting the response to protamine zinc insulin, it was felt that those patients, who by the character of their response possessed an available supply of endogenous insulin, would be adequately controlled by diet with or without a single daily dose of protamine zinc insulin. Those who showed a lack of endogenous insulin would not be controlled in this simple manner.

When put into use, the method consisted in having the fasting patient appear in the morning for three blood specimens to be taken at three-hour

intervals. Studies on 50 diabetic persons were carried out. The results, together with the type of therapy that controlled the diabetes adequately, are presented in the accompanying tables. The fifteen patients in Table I range in age from 41 to 66 years. The highest initial blood sugar level is 280 and the lowest is 80 mg. per cent. All show a descending level from the initial determination, indicating an ability to produce endogenous insulin; hence a mild degree of diabetes controllable by diet alone.

Patients 3, 7, 8, and 15 show figures altogether within normal limits. These patients had been on restricted diets for varying periods, and the diabetes was controlled before the tests were performed. Here the results are similar to those in normal persons and indicate only adequacy of control.

An estimate of the severity of the disease can obviously not be made when the test is performed on patients under treatment. This is further borne out in Table II. Patient 49 was being transferred from regular insulin to protamine zinc insulin, and at the time of the test was receiving 25 units protamine zinc and 10 units standard insulin. While the curve descends from a high initial level, indicating a mild grade of diabetes controllable by protamine zinc insulin alone, it has obviously been influenced by the administered insulin and fails to give a true picture. This patient has actually required the combined use of standard and protamine zinc insulin for control. The second patient, receiving 20 units protamine zinc insulin daily, shows figures within the normal limits, the curve simulating that of the normal person and indicating satisfactory control by diet combined with one dose of protamine zinc insulin.

Table III includes 19 patients showing patterns in which the curve descends from an initial level higher in most instances than those in Table I. All these patients were properly controlled by diet combined with one daily injection of protamine zinc insulin. An exception is noted in the case of patient 30, whose three-hour level is higher than the initial determination. This patient was in the hospital recovering from acute pyelitis. It is probable that the infection distorted the curve, giving it the appearance of that found in a person with severe diabetes. Patients 19, 20, and 27 present flat curves, with none of the values much higher than the normal. These patients have a mild form of diabetes controllable by diet alone. A single daily dose of protamine zinc insulin is being given to keep them aglycosuric since they refuse to stay within dietary bounds. Here the studies, having established the mildness of the disease and its potential control by simple measures, serve as an aid in detecting dietary disobedience.

In Table IV are 12 patients ranging in age from 20 to 82—5 being below age 40. Patients 35, 36, 37, and 38 show a uniformly ascending type of curve. Patients 39 and 40 show the first two figures of equal value and the third lower. Patient 41 shows the last two figures higher than the first. Patients 42, 43, and 44 show the middle figure highest. Patients 45 and 46 show the middle figure lower than the others. None of these patients could be controlled by diet alone or combined with a single dose of protamine zinc insulin.

TABLE III

NO.	PATIENT	AGE	BLOOD SUGAR			THERAPY
			INITIAL	AT 3 HR.	AT 6 HR.	
16	E. S.	52	330	166	143	10 PZI
17	Mrs. S. N.	49	390	280	235	25 PZI
18	V. J.	47	250	224	186	10 PZI
19	L. A.	47	130	100	92	10 PZI
20	L. S.	55	133	130	130	15 PZI
21	Mrs. A. W.	77	186	140	100	15 PZI
22	A. R.	73	250	228	200	10 PZI
23	Mrs. E. B.	73	305	280	280	10 PZI
24	Mrs. M. R.	66	266	228	190	30 PZI
25	Mrs. R. L.	66	225	218	180	10 PZI
26	S. K.	59	200	172	150	20 PZI
27	S. R.	59	133	133	133	10 PZI
28	S. D.	46	500	400	235	30 PZI
29	A. P.	32	500	450	400	40 PZI
30	Mrs. R. R.	59	345	400	330	15 PZI
31	Mrs. L. W.	60	360	270	220	40 PZI
32	Mrs. E. B.	52	210	180	133	20 PZI
33	G. B.	14	270	228	220	30 PZI
34	J. B.	59	133	122	111	10 PZI

TABLE IV

NO.	PATIENT	AGE	BLOOD SUGAR			THERAPY
			INITIAL	AT 3 HR.	AT 6 HR.	
35	Mrs. R. L. B.	20	520	600	620	25 PZI
36	F. L.	36	360	450	500	20 Standard
37	Mrs. K. S.	49	250	290	330	40 PZI
38	Mrs. J. R.	58	138	160	200	10 Standard
39	R. H.	66	380	380	166	25 PZI
40	R. K.	37	200	200	160	10 Standard
41	C. C.	34	214	266	266	20 Cryst.* in A.M.
42	Mrs. E. F.	50	136	166	154	10 Cryst. in P.M.
43	Mrs. E. A.	40	275	350	255	30 Cryst. in A.M.
44	W. S.	70	250	280	210	20 Cryst. in P.M.
45	Mrs. M. S.	59	200	166	210	20 PZI
46	Mrs. B.	82	400	260	400	10 Standard

\*Zinc insulin crystals.

TABLE V

NO.	PATIENT	AGE	BLOOD SUGAR			THERAPY
			INITIAL	AT 3 HR.	AT 6 HR.	
47	Mrs. H. S.	39	152	150	140	Diet
48	Mrs. E. W.	35	100	99	100	Diet

All required in addition a varying amount of regular insulin. Patients 40 and 41 had mild hyperthyroidism. In both, better control was maintained through the use of two daily doses of zinc insulin crystals.

It is evident that despite the variety in patterns presented by this group not a single one resembled the flat curve of the normal person or the descending curve of the person with mild diabetes. All simulated to some extent the ascending curve of the person with severe diabetes.

Two patients with symptoms of acromegaly and diabetes of pituitary origin are presented in Table V. Both show a flat type of curve resembling the normal, patient 47 being at a higher level. This patient is successfully controlled by diet alone. The second patient originally required insulin for adequate control, but within the past year has required neither insulin nor a severely restricted diet. The test was made early in the remission of the diabetes and is certainly normal in appearance. No conclusion of note can be drawn from these data—excepting that the figures verify the observed mildness of diabetes associated with acromegaly.

#### SUMMARY AND CONCLUSIONS

A knowledge of the degree of severity in diabetes is important for predicting the manner of response to therapy. The person with mild diabetes with a fair supply of endogenous insulin is readily controlled by diet alone or combined with a single daily dose of protamine zinc insulin. The person with severe diabetes, with little or no endogenous insulin, is not so easily controlled, requiring regular insulin in addition to the protamine zinc insulin.

A test for gauging the severity of diabetes has been described here. This consists in making three blood sugar estimations at three-hour intervals on the fasting patient while he is ambulatory. The resulting curves fall into three groups: (1) a continuous descent from the initial reading, all levels being not far above the normal values; (2) a continuous descent from the initial reading, with all levels considerably higher than the normal values; and (3) a group of curves, none of which show a continuous descent, with at least one of the last two readings being higher than the initial value. In the first two groups are the persons with mild diabetes that are readily controlled by diet alone or combined with a single dose of protamine zinc insulin. In the third group are the persons with severe diabetes controlled only with more than one dose of insulin.

Of 50 persons with diabetes studied, 15 fell into the first group, 19 into the second, and 12 into the third. Two patients with acromegaly and diabetes showed flat curves, indicating a mild degree of diabetes since they simulated the response of the normal nondiabetic person. The remaining two patients were tested during treatment with insulin. One, receiving both regular and protamine zinc insulin, showed a descending curve typical of mild diabetes, when, in fact, his was a severe diabetes requiring both types of insulin for control. The other, receiving a single daily dose of protamine zinc insulin, showed a flat type of curve resembling the normal. Four similar curves were noted in the group of patients with mild diabetes on restricted diets for vary-

ing periods before testing. This experience indicates that the test is not applicable to patients under treatment—since in these it offers only a measure of the adequacy of treatment.

The test appears to be most useful in the new or untreated patient with diabetes. By gauging the severity of his disease, a greater degree of efficiency, with economy of time and expense, may be attained in planning therapy.

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### OBSERVATIONS ON A SULFANILAMIDE SOLUTION

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A SOLUTION of sulfanilamide for oral administration, as profound in its action as other forms of sulfanilamide and with no greater tendency to produce side actions, is desirable because of the ease with which it may be administered, especially to children, and the accuracy with which dosage can be measured. Such a solution has been prepared and subjected to a series of experimental tests to determine its clinical applicability. The solution\* is essentially a mixture containing in each fluidounce approximately 30 grains of sulfanilamide and 4 fluid drams of systemic alkalizer (approximately 29.7 per cent sodium lactate and 10.6 per cent potassium citrate by weight) and glucose.

Three separate series of experiments were conducted, the first two employing the entire solution, and the third a solution made up without potassium citrate but otherwise identical with that used in the first series.

#### METHOD OF STUDY

In order to determine tolerability, toxic dosage, and minimum lethal dose, 27 guinea pigs, averaging 230 Gm. in weight, were employed. Increasing quantities of the sulfanilamide solution were administered by stomach tube, in divided doses, twice a day for periods of at least twenty-one days.

\*The solution of sulfanilamide, known as sulfanilamide with citra-lactate, was furnished through the courtesy of Donley-Evans & Co., St. Louis.

A second study was conducted with 7 rabbits, employing the sulfanilamide solution, sulfanilamide powder, and prontosil, all in sulfanilamide equivalent dosage, in order to evaluate them comparatively. The drugs were all administered in divided doses at the same time daily. The blood concentration of free sulfanilamide was determined one, four, eight and ten hours after administration, using the method of Marshall<sup>1</sup>. The residual blood concentration of free sulfanilamide in milligrams per cent was determined eighteen to twenty-four hours after administration of the various preparations used.

Microscopic sections of the brain, liver, stomach and kidneys of the guinea pigs and rabbits, which had been given lethal doses of the sulfanilamide solution, were examined for evidence of pathologic changes in these organs.

In the third series of experiments sulfanilamide solution without potassium citrate was employed. Six guinea pigs, averaging 251 Gm. in weight, were each given 12 c.c. of the solution (representing approximately 12 grains of sulfanilamide) per day in three divided doses, or approximately 3.2 Gm. per kilogram per day, for a period of forty days. Another group of 6 guinea pigs, averaging 247 Gm. in weight, received 15 c.c. each of the sulfanilamide solution daily in three divided doses, or approximately 4.0 Gm. per kilogram per day. These experiments were conducted to determine the tolerance dose of the sulfanilamide solution without potassium citrate.

#### RESULTS

In the first series of experiments it was found that the guinea pigs tolerated 14.8 c.c. of the sulfanilamide solution (approximately 1 Gm. of sulfanilamide) per kilogram of body weight per day for periods of at least twenty-one days, without manifesting symptoms of toxicity or loss in weight. The solution was found to be no more toxic than sulfanilamide powder in equivalent dosage.

When given in doses of 17.3 c.c. (approximately 1.17 Gm. of sulfanilamide) per kilogram of body weight per day, the sulfanilamide solution produced very mild toxic symptoms, but the animals survived this dose for periods of at least twenty-one days. Toxic symptoms with 21.7 c.c. (approximately 1.47 Gm. of sulfanilamide) of the solution per kilogram of body weight per day did not occur when the potassium citrate was removed from the solution. The lethal dose, as determined by this series of experiments, was approximately 26 c.c. per kilogram of body weight (approximately 1.76 Gm. of sulfanilamide). Halpern<sup>2</sup> states that the guinea pig is about as susceptible to the toxic effects of sulfanilamide as is the rabbit. Halpern and Mayer<sup>3</sup> state that the toxic dose for the rabbit is 2 Gm. per kilogram. Raiziss<sup>4</sup> and co-workers find that 2 Gm. administered orally is lethal for rabbits. The findings of one of us (F.L.), in a comparison made at the same time, was that the lethal dose of sulfanilamide in guinea pigs was approximately 1.56 Gm. per kilogram per day.

Chart 1 demonstrates the time of appearance of free sulfanilamide in the blood when administered in the form of solution, powder, and prontosil, in sulfanilamide equivalent doses. It may be observed that free sulfanilamide

appears in measurable quantities in the blood of rabbits somewhat more quickly when given in the form of the solution than when administered in other forms; also, that the solution leads to an appreciably higher concentration of sulfanilamide in the blood than does sulfanilamide powder or prontosil.

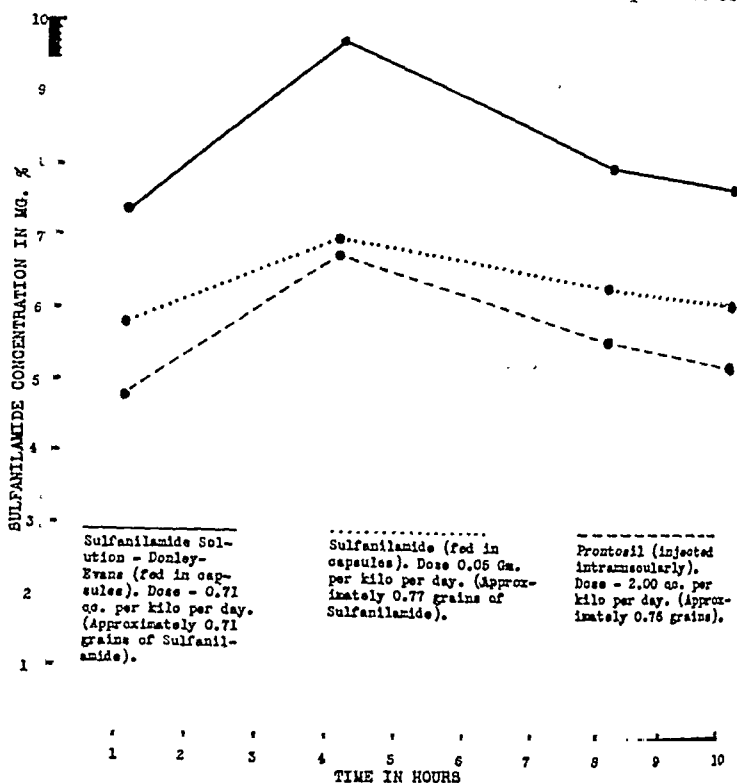


Chart 1.—Blood concentration tests of three forms of sulfanilamide in rabbits.

Chart 2 shows the rate of elimination of sulfanilamide from the blood when administered in various forms. The rate of elimination is no more rapid when the drug is given as the solution than when given in other forms. Since the blood concentration with the solution is somewhat higher than with the powder or prontosil, and since the rate of elimination from the blood is no more rapid with the solution, the residual blood concentration after withdrawal of the drug remains somewhat higher with the solution than with the other two drugs. This fact may indicate that the extent and duration of its action are increased when sulfanilamide is administered in the form of the solution.

The microscopic sections of the brain, liver, stomach, and kidneys of the guinea pigs and rabbits given lethal doses of the sulfanilamide solution revealed no evidence of disease in these organs. There were no significant pathologic changes either in the organs of animals dying within a few days after the administration of large toxic doses or in the organs of animals given mildly toxic doses for periods up to twenty-one days.

In the third series of experiments, the series employing sulfanilamide solution without the potassium citrate, animals given 12 c.c. of the solution per



day in three divided doses made a continuous gain in weight and showed no toxic symptoms. When the dose was increased to 15 c.c., two of the animals died within the first three days; the other four survived until the experiment was concluded at the end of thirty days. Although these animals did not gain weight, they retained their original weight fairly well. They developed mild toxic effects, such as drowsiness and weakness in the hind legs, with some stiffness.

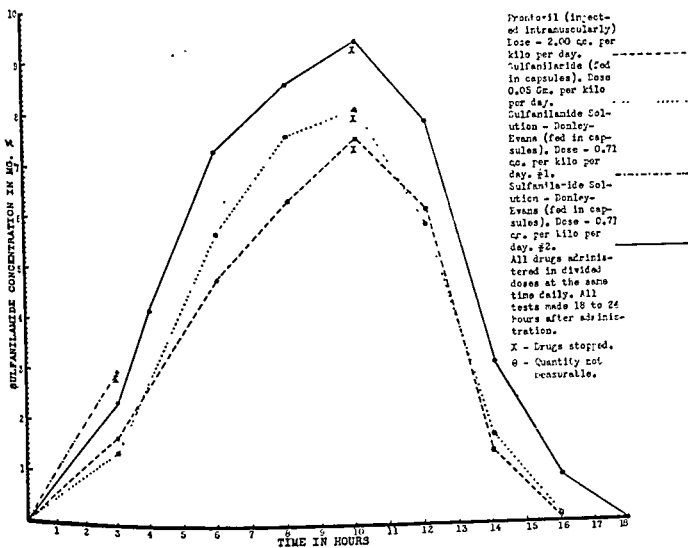


Chart 2.—Residual blood concentrations 18 to 24 hours after administration in milligram per cent.

It appears, therefore, that the sulfanilamide equivalent tolerance dose of the sulfanilamide solution, without potassium citrate, is slightly above 3.2 Gm. per kilogram of body weight per day when given in three divided doses by stomach tube. The administration of 10 c.c. of the solution containing potassium citrate to a 250 Gm. guinea pig apparently exceeds the tolerance dose of potassium citrate for guinea pigs. Hence, it seems that in the guinea pig, symptoms of intolerance of sulfanilamide solution containing potassium citrate are probably due, at least in part, to the large amount of potassium citrate present.

Clinical experience with sulfanilamide solution has confirmed the experimental evidence demonstrating its safety, and the extent and duration of its action. Thus far, after extensive clinical trial, no alarming toxic reactions have been observed. It is our impression that the side actions are definitely more numerous with sulfanilamide in the form of the dry drug than with the solution, when equal doses of the drug are administered.

## CONCLUSIONS

1. In the guinea pig and rabbit higher blood concentrations of free sulfanilamide are obtained when the drug is given as the solution than when it is administered in equivalent doses in other forms.
2. The rate of elimination of the sulfanilamide is no more rapid when given as the solution than when given in other forms.
3. The sulfanilamide appears in the blood more rapidly when given as the solution than when administered in other forms.
4. Sulfanilamide solution, therefore, may be expected to be as effective in the treatment of certain infections as any other form of sulfanilamide.
5. From the experimental evidence presented, it can be concluded that there are probably fewer toxic side actions with the sulfanilamide solution than with sulfanilamide in the form of the dry drug when administered in sulfanilamide equivalent dosage.

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## THEOPHYLLINE WITH ISOPROSPANOLAMINE IN HEART DISEASE\*

## WITH ESPECIAL REFERENCE TO CONGESTIVE FAILURE

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THE treatment of congestive heart failure remains a challenge to the cardiologist's therapeutic ingenuity. Venesection has proved by experience to be a lifesaving measure in congestive failure, but it is too seldom used in our modern techniques. This procedure lessens the peripheral cardiac load and enables more efficient myocardial function. The lessening of pressure in the venous circulation is temporary but frequently of sufficient duration to enable the weakened right heart to regain compensation. A drug capable of lowering venous and spinal fluid pressures, as well as producing capillary dilation, would considerably enhance the value of venipuncture. Our investigations suggest the fulfillment of these criteria in theophylline with isopropanolamine. This is a water-soluble theophylline compound, and represents a combination of theophylline with isopropanolamine.<sup>1</sup> The dilation of the capillaries of the nail bed, as well as lowering of the venous and spinal

\*From the service of William Egbert Robertson, M.D., Philadelphia General Hospital.  
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TABLE I

TABULATION OF AVERAGE CHANGES AND THEIR PROBABLE ERRORS

average readings of 21 patients studied after the intravenous administration of 10 c.c. of 0.025 per cent theophylline with isopropanolamine.

	IMMEDIATE		5 MIN.		10 MIN.		15 MIN.	
	AVG.	PROBABLE ERROR	AVG.	PROBABLE ERROR	AVG.	PROBABLE ERROR	AVG.	PROBABLE ERROR
al fluid pressure	-22	±5.0	-28	±5.0	-36	±5.5	-24	±7.0
pressure	-14	±6.5	-9	±4.5	-20	±9.0	-4	±5.0
diastolic arterial pressure			-8	±3.0	-14	±4.0	-10	±0.6
systolic arterial pressure			-3	±1.0	-4	±3.0	-10	±0.6
illary reading	+6	±2.0	+10	±1.0	+10	±2.0	+8	±1.0

first, finally diminishing considerably as the capillary dilatation persists. The end of the ten-minute period marks the greatest lowering of systolic pressure, which then rises with the elevation of venous and spinal fluid pressures.

These observations are in accord with current physiologic concepts. It is well established that spinal fluid pressure fluctuates in direct proportion to arterial pressure, and variations of diastolic pressure are similarly closely related to the tone of the peripheral capillary bed.

## SUMMARY

The effect of theophylline with isopropanolamine was studied in a series of 21 patients, with reference to capillary dilatation, arterial, venous, and spinal fluid pressures. These pressures showed a definite fall associated with capillary dilatation. This response to the drug, although comparatively slight, may prove useful in the treatment of congestive heart failure in conjunction with venesection and other appropriate measures. Further study may show a more sustained effect with the oral or intramuscular administration of this preparation. Our observations showed considerable relief of pulmonary congestion and orthopnea shortly after injection, lasting from three to five hours. The clinical improvement was far more sustained than the laboratory indicate.

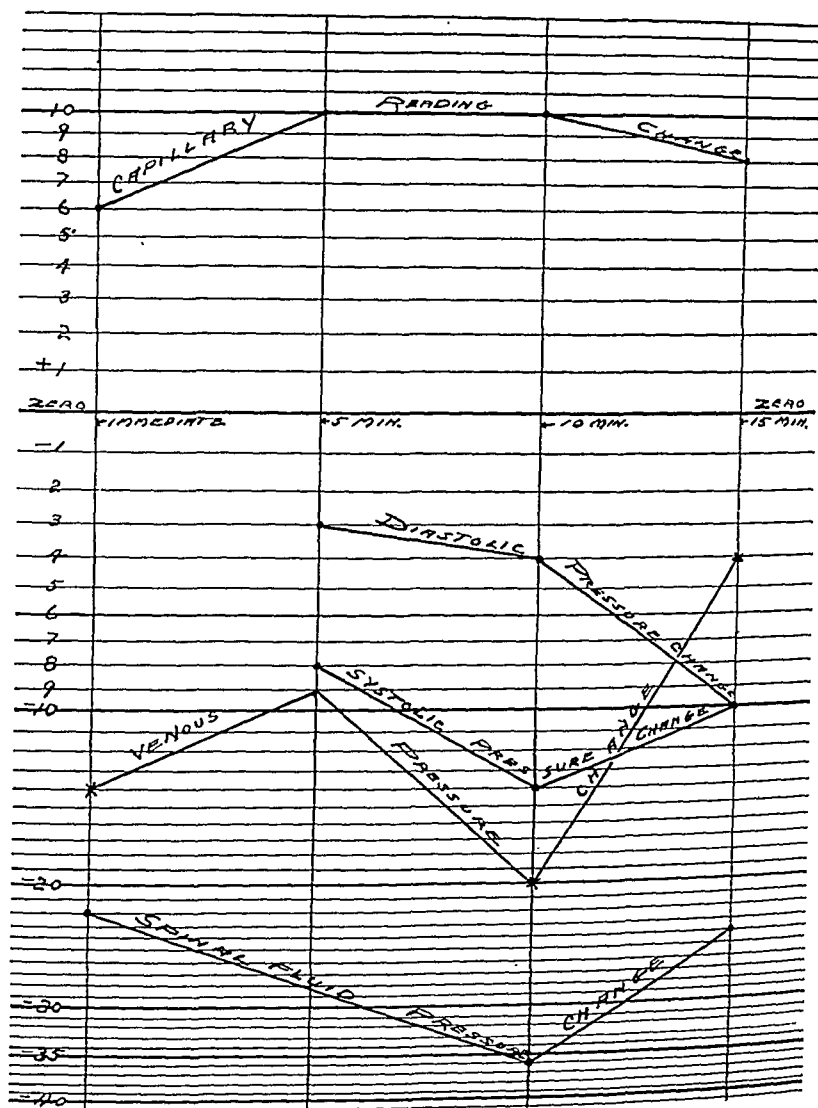


Fig. 1.—Arith-log study of average changes of pressures and capillary readings of micrometer after the intravenous administration of 10 c.c. of 0.025 per cent theophylline with isopropanolamine.

Table I shows a tabulation of the average changes, with the probable error at the four observed points for all the experiments. This indicates a fall of all the pressures with a commensurate dilatation of the capillaries in the fingernail fold. The greatest change occurred ten minutes following the injection. These results were plotted on arith-log paper (Fig. 1), utilizing the suggestion of Pearl<sup>6</sup> that variables showing a rate of change must be coordinated to a base to establish the respective relationship. It is to be noted that the rate of change of venous and spinal fluid pressures from immediately after administration to the ten-minute period are almost parallel. The rate of capillary dilatation rises rapidly, simultaneous with the fall in spinal fluid and venous pressures during the first five minutes. It then remains constant and gradually returns to normal. The diastolic pressure falls gradually

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	AVG.	PROBABLE ERROR	AVG.	PROBABLE ERROR	AVG.	PROBABLE ERROR	AVG.	PROBABLE ERROR
Spinal fluid pressure	-22	±5.0	-28	±5.0	-36	±5.5	-24	±7.0
Venous pressure	-14	±6.5	-9	±4.5	-20	±9.0	-4	±5.0
Systolic arterial pressure			-8	±3.0	-14	±4.0	-10	±0.6
Diastolic arterial pressure			-3	±1.0	-4	±3.0	-10	±0.6
Capillary reading	+6	±2.0	+10	±1.0	+10	±2.0	+8	±1.0

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## SUMMARY

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We wish to express our gratitude to the National Drug Co. for the material supplied for the research problem.

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## HYPERGLOBULINEMIA IN GRANULOMA INGUINALE

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**G**RANULOMA inguinale and lymphogranuloma inguinale, the so-called fourth and fifth venereal diseases, are quite distinct clinical entities in spite of the similarity of their names. The former is essentially a disease of the skin and corium<sup>1</sup> and seldom invades the deeper tissues. Beginning as a small macule on penis or vulva, it spreads as a superficial destructive fungating mass over the pudendum, perineum, and inguinal regions. Scrapings from the mass are characterized by the pathognomonic Donovan bodies. The infection usually responds satisfactorily to tartar emetic injections or, better still, to Fuadin therapy.

Lymphogranuloma inguinale, on the other hand, is essentially a disease of the lymphatic tissues. Beginning as a small macule on the pudendum, it develops deep-seated buboes with areas of induration and softening that later discharge through multiple fistulas. Involvement of the rectum with stricture is characteristic, and the Frei test appears to be pathognomonic. There is no satisfactory specific therapy.

Lymphogranuloma inguinale is almost certainly a virus infection.<sup>2</sup> The nature of the Donovan bodies which have been demonstrated to be the cause of granuloma inguinale<sup>3</sup> is still a matter of dispute, although there is much to be said in favor of Donovan's original contention that they are protozoan in character.

In 1936 Gutman and his co-workers reported a series of serum protein determinations in lymphogranuloma inguinale, their reports, however, being only incidental to more extensive studies. In their first communication,<sup>4</sup> they discussed 12 cases of lymphogranuloma inguinale, all with a positive Frei test. Serum globulin values, ranging from 2.2 to 7.8 per cent, were found; in all but 2 cases the values were higher than 4.1 per cent. Half of the cases had a serum globulin content of 5.0 per cent or higher. In a second series<sup>5</sup> they report 13 cases, all in Negro women with positive Frei reactions; 12 of the 13 were under treatment for rectal stricture. The serum globulin findings in this series were essentially like those in the first; euglobulin determinations ranged from 0.7 to 4.0 per cent, being generally, but not constantly, proportionate to the total globulin values. In a third communication,<sup>6</sup> they reported 35 cases of lymphogranuloma inguinale, all with positive Frei test. The total serum globulin ranged from 2.2 to 8.3 per cent, being 5.0 per cent or higher in nearly half the cases, and above 4.0 per cent in three quarters. The euglobulin ranged from

0.6 to 4.0 per cent, again being generally, but not always, proportionate to the total serum globulin. Similar findings were reported by Rosen, Rosenfeld, and Krasnow.<sup>7</sup>

The foregoing findings indicate that hyperglobulinemia is a characteristic feature of lymphogranuloma inguinale and suggest that it might help to distinguish it from granuloma inguinale in those borderline cases in which confusion is possible. It seemed worth while, therefore, to look through our files for cases of granuloma inguinale in which blood protein determinations had been made. Seven such cases were found, 6 of them florid, the seventh nearly healed following tartar emetic injections. In some of these the diagnosis had been confirmed by the presence of Donovan bodies; in all of them the picture was quite characteristic. The findings are shown in Table I.

TABLE I

	TOTAL PROTEINS %	SERUM ALBUMIN %	SERUM GLOBULIN %	EUGLOBULIN %	PSEUDO- GLOBULIN %
G. V. (florid)	9.3	3.9	5.4	1.7	3.7
J. H. (florid)	9.40	3.72	5.68	1.18	4.50
S. W. (florid)	8.5	4.6	3.9	1.9	2.0
G. H. (florid)	10.3	4.9	5.4	1.3	4.1
R. W. (florid)	8.25	3.81	4.44	0.90	3.54
P. R. (florid)	8.1	3.4	4.7	1.7	3.0
P. R. (treated)	7.55	4.57	2.98	0.15	2.83

A much larger series would obviously be required to determine the uniformity with which hyperglobulinemia occurs in granuloma inguinale. The interesting feature is the extremely high euglobulin percentage with a somewhat more moderate increase in the total serum globulin.

## SUMMARY

Recent reports in the literature indicate that hyperglobulinemia is present in most cases of lymphogranuloma inguinale.

The same phenomenon occurs to a marked degree and with high euglobulin values in granuloma inguinale.

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## THE OCCURRENCE OF GUANIDINE-LIKE SUBSTANCES IN THE BLOOD IN ESSENTIAL EPILEPSY\*

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NUMEROUS studies of the concentration of guanidine-like substances in the blood have been carried out in the past ten years by various investigators in both normal and pathologically affected subjects, as well as in animals manifesting tetanic or convulsive symptoms, renal and hepatic involvements. A field, apparently missed by previous investigators, in which increase in the guanidine-like substances in the blood might be expected, is represented by the essential epilepsies—especially when the seizure pattern is that of *grand mal*.

Fifteen institutional patients were studied by the colorimetric method of Pffifner and Myers, as modified by Andes and Myers,<sup>1</sup> except that, instead of the final values being read ocularly, more objective readings were taken from a photoelement photospectrometer, with monochromatic light (485 m $\mu$ ) placed at our disposal in the Kettering Laboratory. This was deemed desirable because (a) Lambert-Beer's law is valid only for monochromatic light; (b) the color of the blank is relatively great compared to the color developed by the guanidine; (c) finally because the maximum absorption of both occurs in a region of low sensitivity to the human eye.

Basal levels were first determined in each case (the average of five taken during apparently normal periods), then serial determinations made during aura, seizure, and one hour after cessation of convulsion. The findings are presented in Table I. These figures show that in the patients with essential epilepsy studied, guanidine content of the blood rises significantly. Blood guanidine level rose during the aura, reached its highest point during the seizure, then dropped back to basal level.

With the foregoing facts demonstrated it becomes possible to speculate on the place of guanidine in the epileptic pathology. We emphasize, however, that though all the cases studied showed the same tendencies, the total number is still small.

Experiment shows that an increase in blood guanidine level is followed by convulsions. Guanidine has powerful action on the neuromuscular junction. The response of the muscle to single shock nerve stimulation is modified as the guanidine concentration is increased. Instead of being followed by a single response, a tetanus is now produced, sustained at times for minutes.

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TABLE I  
GUANIDINE VALUES  
(Expressed in Milligrams Per Cent)

PATIENT NO.	AVERAGE BASAL LEVEL	AURA	CONVULSION	POST CONVULSION
1	0.50	0.80	1.60	0.61
2	0.41	0.76	1.31	0.53
3	0.37	0.64	1.17	0.51
4	0.31	0.60	0.76	0.50
5	0.45	0.74	1.47	0.56
6	0.38	0.69	1.21	0.54
7	0.42	0.67	1.14	0.54
8	0.36	0.53	0.93	0.51
9	0.42	0.66	1.26	0.52
10	0.47	0.79	1.50	0.58
11	0.47	0.80	1.57	0.52
12	0.37	0.62	0.92	0.51
13	0.30	0.55	0.80	0.50
14	0.49	0.73	1.32	0.57
15	0.5	0.79	1.54	0.61

Thus with sufficient increase in guanidine concentration, any "normal" stimulus may peripherally be so effective as to cause convulsion. Clinically guanidine rise may come from any of the following sources: increased formation; decreased destruction by liver; decreased elimination by kidney. The shape of the curve would tend to indicate the first.

#### SUMMARY

1. In the instances of essential epilepsy studied, the basal content of guanidine in the blood was found significantly high.

2. All who presented convulsions of the *grand mal* variety showed a blood guanidine rise during the aura reaching a high point during convulsion.

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# LABORATORY METHODS

## AN APPARATUS FOR MULTIPLE ESTIMATIONS OF CHOLESTEROL IN OXIDATIVE MICROMETHOD\*

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RECENTLY I reviewed modifications and factors in the oxidative technique for the microanalysis of blood lipids.<sup>1</sup> In the present study I wish to describe a special apparatus that has proved useful in the oxidative micromethod for blood cholesterol.

The digitonin precipitate of cholesterol requires filtration, washing, and redissolving in boiling methyl alcohol. An apparatus has been devised for this purpose, similar in principle to that previously described,<sup>1</sup> but containing a number of technical improvements and permitting the simultaneous estimation of up to 12 samples; more could be added by enlarging the apparatus. A photograph of the entire apparatus is shown in Fig. 1, and a close-up of two units in Fig. 2.

The framework consists of a base 2 feet wide, 4 feet long, and  $\frac{7}{8}$  inches thick, set on two crossboards that act as feet and add support. On the back of this base is set a box section, 14 inches wide and 6 inches high. The top of the box section is made of two halves of equal width, the back half being permanently attached to the box section and the front half attached with removable screws (Fig. 2). At intervals circles are cut out along the center of the top of the box section, half of the circle being in the front of the back half and the other half of the circle being in the back of the front half of the top of the box section. These circular holes are made large enough to accommodate snugly the neck and upper part of 1 liter, side-necked, filtering flasks, which sit upon the base within the box section. The diameter of these holes will depend upon the shape of the flasks and the thickness of the wood used in making the top of the box section: a diameter of  $3\frac{1}{4}$  inches was used in making the apparatus illustrated in Fig. 1. The removable front half of the top of the box section enables these filtering flasks to be removed if broken or for emptying.

An upright board, 3 inches high, is placed  $1\frac{1}{2}$  inches forward from the back edge of the top of the box section. Running along the back of this upright board is a half inch iron pipe, interrupted by T-joints at intervals; to these joints are attached short lengths of pipe that extend forward through the upright board. On the front of these extensions is a compressed air tap, connected by thick-walled rubber tubing to the side neck of the filtering flasks. The extreme left end of the pipe (the reader's left when looking at the apparatus) is closed. The right end is brought through the upright, and another compressed air tap is attached. This latter tap connects by means of heavy-walled tubing to a Hyvac

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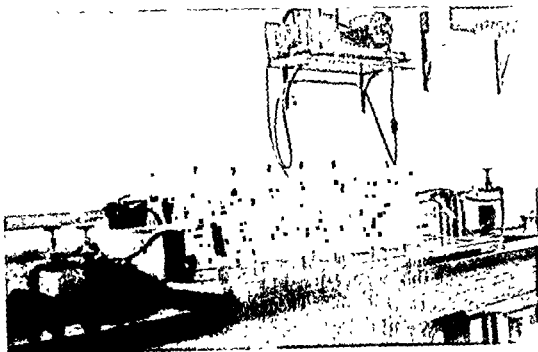


Fig. 1.—A general view of the cholesterol apparatus.

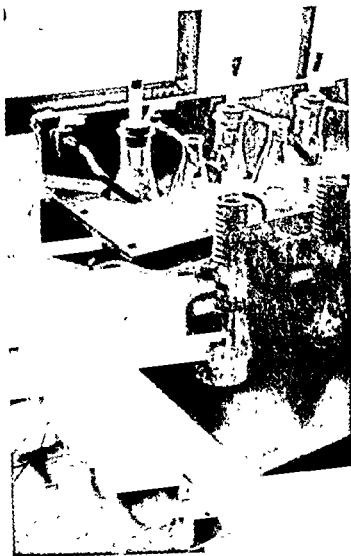


Fig. 2.—A close-up of two units of the apparatus.

pump placed on a shelf attached to the wall in a convenient place just above the apparatus. The apparatus shown in Fig. 1 was originally designed to function with a water pressure filter pump, and an attachment to such a pump through a trap was included in the apparatus; these are shown to the right in Fig. 1. The water pump, being too light, was discarded in favor of the more satisfactory Hyvac pump.

The stem of the sintered glass filters (4G4, Schott and Jen, Jena) is fitted into a No. 0, one-holed rubber stopper, which in turn is inserted into the top of a 15 ml. centrifuge tube that passes through a rubber stopper in the top of the filtering flasks. The bottom of the centrifuge tube is cut off. When appropriate taps are open, from 1 to 6 cholesterol digitonide precipitates may be filtered at one time along the back row of filters.

After the cholesterol digitonide has been filtered free of its mother liquor and the precipitate washed with acetone and ether in the back row of filters, it is transferred in the sintered glass filter with attached rubber stopper to the front row of steam coils for redissolving. Another batch of cholesterol may then be started filtering. Live steam enters the coils from the left of the apparatus through copper tubing, arranged in 6 coils of sufficient diameter to enclose snugly the sintered glass filters. The copper tubing is continuous from one steam coil to the other, and the whole is attached to the front of the box section at a convenient height to permit the oxidation flasks to be inserted and removed. The extreme right end of the steam line empties into a sink. Underneath, and fitting up into the bottom of each steam coil, is a glass suction head made by a glass blower. The top of the glass suction head contains a flanged opening large enough to receive the No. 0 rubber stoppers of the sintered glass filters. There is a side neck attached through thick-walled rubber tubing to a compressed air tap held against the front of the box section. All these taps are connected by piping along the inside of the front of the box section; this piping ends at the right by coming forward through the front board of the box section and is sealed there with another compressed air tap. This latter tap is connected to the tubing running to the Hyvac pump by thick-walled rubber tubing and a T-tube. Suction may be directed either to the front or to the back rows or to both by opening and closing appropriate taps at the right end of each pipe line. T-tubes and clamps attached in the rubber tubing going to the Hyvac pump act as release valves.

The glass suction head is sufficiently wide at the base ( $1\frac{3}{8}$  inches) to fit over the neck and top of 125 ml. glass-stoppered Erlenmeyer flasks (the oxidation flasks). To make an airtight joint with the oxidation flasks, strips of flat rubber tubing,  $1\frac{3}{4}$  inches wide are doubled over the bottom of the glass suction heads. Each suction head is then attached loosely into pliable metal strip clamps fixed to the front of the box section.

#### ESTIMATION OF BLOOD CHOLESTEROL

The technique of estimating blood cholesterol is summarized here. A more detailed description and discussion, directions for the preparation of reagents, and modifications necessary for special variations have been given in a previous report.<sup>1</sup>

One milliliter of whole blood, serum, or plasma is run slowly into 25 ml. of alcohol-ether solution, 3:1, both redistilled. The proteins are filtered off, washed several times with the extracting fluid, and finally pressed with a cleaned glass rod. The combined filtrates are boiled down to a volume of about 20 ml. in an Erlenmeyer flask, 0.1 ml. of saturated sodium hydroxide is added, and the flask is placed on a steam bath at a temperature sufficient to evaporate the extract to a volume of 1 ml. in thirty to sixty minutes. One milliliter of 25 per cent sulfuric acid and a few drops of phenol red indicator are added, and the flask is placed on top of the steam bath until patches of condensed water vapor begin to appear on the side of the flask. The aqueous layer is then extracted four to six

times with 5 to 10 ml. portions of petroleum ether. Five milliliters of 0.2 per cent digitonin (Merck) in 50 per cent alcohol are added to the combined petroleum ether extracts, and the mixture is concentrated almost to dryness. Ten milliliters of distilled water are added to the precipitated cholesterol digitonide and brought to a gentle boil. After cooling, 10 ml. of acetone are added, and the mixture is shaken. The cholesterol digitonide is then filtered in the apparatus here described, the precipitate is washed in succession with acetone and ether, and is then redissolved in three successive portions of boiling methyl alcohol, each portion being drawn with suction into a 125 ml. glass stoppered Erlenmeyer flask. The methyl alcohol solutions are then evaporated to dryness without burning on a hot plate, the 125 ml. Erlenmeyer flask is transferred to a steam bath, and the last trace of methyl alcohol vapor is blown off with a gentle stream of air. The cholesterol digitonide is then oxidized with 5 ml. of Nieloux reagent and exactly 3 ml. of 1 N potassium dichromate, and heated in an oven for thirty minutes at 124° C. The flask is removed from the oven, and about 75 ml. of ice-cold distilled water are added. The unreduced potassium dichromate is then titrated with standard 0.1 N sodium thiosulfate. The figure thus obtained is subtracted from that of a blank treated in the same manner, but without the original blood, serum, or plasma. The difference is expressed in terms of exactly 0.1 N potassium dichromate, divided by 10.61 and multiplied by 100 to give the number of milligrams of cholesterol per 100 ml. of blood, serum, or plasma.

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## A STANDARDIZED MASTIC TEST FOR SPINAL FLUID\*

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**S**ENSITIVITY and *specificity* are the two qualities that characterize the value of a serologic test. But a third quality, *constancy*, is important too. In syphilis the difficulties with regard to constancy have been overcome to a large extent, as far as competent laboratories are concerned. This, however, cannot be said regarding the invaluable Lange test and its modifications, the colloid tests of the spinal fluid.

Many formulas have been worked out to guarantee reagents of a constant quality, but none of them is fully satisfactory. For this reason, the colorful reagent of Lange<sup>1</sup> has been replaced to a certain extent by other colloids. Among these, mastic (Emanuel<sup>2</sup>) and other gums have the first place. The resins offer a great advantage since, in preparing the colloids, the procedure can be based on well-known physical factors which are the foundation of serology in syphilis. Sachs and Rondoni have detected the fundamental fact that the reactivity of the Wassermann antigens depends on the speed with which heart extract and saline are mixed. Sachs, as well as Kafka and Jacobsthal,<sup>3</sup> have stated that the same law is valid for the mastic test. As the antigens for the lipid-precipitation tests, too, obey the law of Sachs-Rondoni, the similarity between lipid and colloid tests is especially impressive.

\*From the University of Georgia, School of Medicine, Augusta.

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The speed of mixing the saline with heart extract or water with mastic solution determines not only the sensitivity of such colloids for their use as reagents, but also their faculty to become precipitated by other physical influences, especially by salt solutions. The law of Sachs-Rondoni, as broadened for these different conditions, may be summarized in this way: When a colloidal reagent for syphilis is prepared by mixing an alcoholic solution of heart lipid, resin, etc., with water (or saline), the opacity of the resulting colloid, and, simultaneously, its reactivity toward serum or spinal fluid, increases in the same degree in which the mixing speed during preparation decreases. Toward salt solutions the behavior is the reverse. The colloids are the clearer and the more easily precipitated by salt solution, the faster the mixing had been done. We have the extremes: (1) Slow mixture—dense colloids—high sensitivity for serum and spinal fluid—low sensitivity for salt solutions. (2) Quick mixture—clear colloids—low sensitivity for serum and spinal fluid—high sensitivity for salt solutions. (The reactivity in the Wassermann reaction is substantially identical with the precipitation in the flocculation tests.)

But in practice the determination of the mixing speed is rather inexact. If we request that the mixing be done drop by drop, and be finished within a certain time, the intervals between the single drops (which influence the result in a high degree) cannot be standardized perfectly. In any case, the results with the different modifications of the mastic reaction were unsatisfactory because of a definite lack in constancy.

In the lipid flocculation tests, mixing the reagents by dropping has been substituted for some years by a two-step-mixing. This can be adopted for the mastic test.<sup>4</sup>

#### EXPERIMENT

To a row of tubes containing 1 c.c. of a 1 per cent alcoholic mastic solution, distilled water is added as rapidly as possible, in the amount of 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, respectively. Opacity develops rapidly in all tubes within the first few seconds, then increases slowly. After two minutes no perceptible change is noted, and the tubes show different degrees of opacity. If this state is reached, sufficient water is added quickly to each tube to make 4 c.c. of water. The final effect is read five minutes later.

There is present a series of colloidal solutions, the opacity of which increases from tube to tube to a certain point, and then decreases by degrees. All the colloids contain the same amount of water, alcohol, and mastic. They are all prepared with the same mixing speed, and differ from each other only in the fact that water is added in two portions of different amounts. This influences the final density in a manner similar to that produced by different mixing speeds. The significance of this statement is derived from the fact that the law of Sachs and Rondoni is valid here, too. The densest colloid is the most sensitive to spinal fluid and the most resistant to salt solution. The clearest one has the opposite qualities; it is the least sensitive to spinal fluid and the most sensitive to salt solution.

*Instead of the mixing speed, the amount of the first portion of water is made the deciding factor. It is evident that with this method it is easy to prepare a series of colloids with exact gradation, and to repeat the preparation of the selected one in a constant way. The highest density and the highest sensitivity are present in the tube in which 1 c.c. of water has been added in the first step. We may state that the ratio of one part mastic to one part water is an "optimum." This ratio is a characteristic, distinguishing peculiarity of all solved substances which can be changed into a colloid by adding water. It is, for instance, 1:0.5 for the original Meinicke extract. To the left and to the right of this optimum we have tubes with decreasing opalescence; that is to say, if we add either more or less than 1 c.c. of water, the colloids are clearer and less sensitive.*

One difference, however, exists between the two sections of the series. If the first addition is less than 1 c.c. of water, the peculiarity of the final colloid is not completely determined by the first step, and the speed of the second addition of water is also important in this respect. While after an addition of 1 c.c. or more, the colloidal fate is definite, and the speed of the second step is without distinct influence.

The proper distribution of the whole amount of water between the two portions secures the desired degree of sensitivity. However, we request more than a constant sensitivity. The colloid shall yield a certain constant opalescence, so that even a slight reaction may be noted clearly. In this regard the concentration of the alcoholic mastic solution is the most important point. Usually from a 10 per cent stock solution a 1 per cent routine solution is made. But mastic is a trade product, the composition of which is not fully constant. A various, sometimes considerable, part is insoluble in alcohol. Thus the concentration of the stock and the routine solutions, and of the final colloid, may be lower than expected.

Another still more important requirement depends on the concentration, namely, the correlation between the peak of the curve and the dilutions of the spinal fluid. If a large number of syphilitic spinal fluids is examined with the same reagent, it is striking how constantly the peaks of the curves coincide with the same dilution of the spinal fluid. There are differences between various methods. With colloidal gold the peak of the curve corresponds to the spinal fluid dilution 1:80. With the method for mastic described here the peak of the curve corresponds to spinal fluid 1:8, which is slightly different from the original method and from the "Normomastixreaktion" (Kafka). In each method the different syphilitic curves differ from each other in depth and width, but only exceptionally in the position of the peak of the curve. Even a slight shifting has its significance, provided that such a shifting is not caused by faulty technique.

It is remarkable that the curve is shifting to the right if the concentration of mastic is lowered. To secure constancy of the results the mastic concentration of the colloid must be kept constant by adjusting the dilution with alcohol to the individual specimens of the resin.

The last point to be considered is the balance between the sensitivity to spinal fluid and to sodium chloride. A colloid test that has the proper degree

of sensitivity to the spinal fluid, but is precipitated by the salt solution, is somewhat inconvenient. The use of a salt solution of lower concentration entails some complications which cannot be described here. A small quantity of alkali, as stated by Cutting,<sup>5</sup> and by Kafka and Goebel,<sup>3</sup> raises the resistance against sodium chloride, but influences only slightly the behavior toward spinal fluid.

After one has arrived at these conclusions, the technique is rather simple, if a laboratory is in a position to use a tested resin. A bit more complicated, but without any difficulty for an experienced worker, is the procedure of testing. The following reagents and glassware are needed: (1) Solution of 10 Gm. mastic in 90 Gm. absolute alcohol. After some days the solution is filtered, and the residue is removed. The stock solution can be kept in the ice box without any change in its composition. (2) Absolute alcohol. (3) Distilled water. (4) Aqueous solution of 0.5 per cent sodium carbonate. (5) Tubes 80 mm. long, 10 mm. wide. (6) Racks with 18 tubes in each row. It is convenient to have racks with two rows, one above the other, in such a way that the tops of the lower row of the tubes do not overlap the bottoms of the upper row. (7) Pipettes of 1, 5 and 10 c.c. (8) Graduate cylinders of 10, 25, and 100 c.c. (9) Beakers of 30 and 100 c.c.

*Procedure A (With Tested Resin).*—The routine solution of mastic is made by mixing 1 part of the stock solution with as much absolute alcohol as directed (usually 1 : 12 to 14, resulting in an approximately 0.7 per cent solution). Alkalinized water, with a content of 0.005 per cent sodium carbonate, is prepared by adding 0.1 c.c. of the 0.5 per cent sodium carbonate solution to each 10 c.c. water. For the preparation of the colloidal reagent, 1 part of routine solution is mixed with 4 parts of alkalinized water, the mixture being performed in two steps.

*Example.*—To prepare 50 c.c. colloid, three 100 c.c. beakers are supplied with 10 c.c. routine solution, 10 c.c. alkalinized water, and 30 c.c. alkalinized water, respectively.\* Into the mastic solution, 10 c.c. water is poured as fast as possible and mixed thoroughly by rotating the beaker. A delay in the mixture brings the uncontrollable factor of speed into the procedure. After an interval of two minutes, the contents of the third beaker, 30 c.c., are added. A moderate variation in the speed is without distinct influence in this stage. After five minutes the colloid is ready for use. It does not change within a few hours, and changes very little in several days. For the dilution of spinal fluid 0.5 c.c. sodium chloride (0.9 per cent) is poured into each of 9 tubes, 0.5 c.c. spinal fluid is added to the first tube, and diluted in the usual manner. One-half cubic centimeter of the colloid is used for each tube, and the result is read off the next day (at least sixteen hours later).

*Procedure B (Testing of Mastic).*—Different routine solutions are made by diluting the stock solution with absolute alcohol in different proportions (1 : 10 to 15). From each of these<sup>6</sup> solutions a series of colloids is made by varying the first portion of water in the following manner: To a series of tubes containing 1 c.c. of mastic solution, 1.0, 1.2, 1.4, 1.6 c.c., respectively, of al-

\*This is a proportion between the components frequently found to give the desired sensitivity of the colloid.



kalinized water are added and made up to 4 c.c. water after two minutes. After five minutes the colloids are ready. All colloids are tried on the same negative and positive spinal fluids, and the most satisfactory reagent is selected as a basis for the routine preparation.\* This description is a sketch of our procedure; it is not really intended that all these 24 colloids be tested. Usually the samples of mastic do not differ so much from each other, and the technique may be simplified. From an average routine solution two colloids are prepared by adding, as a first step, 1 and 1½ parts of alkalinized water. According to the results on two spinal fluids, the further testing can be reduced to a few variations. Only for specimens of unusual peculiarities, or for a new kind of resin, is the more extended testing necessary. With the help of this method, reagents have been worked out from 24 different kinds of resin.<sup>6</sup>

A few technical details may be mentioned here. Some specimens of mastic give hyposensitive colloids with the regular technique. The use of 95 per cent alcohol, instead of absolute alcohol, for the preparation of the routine solution raises the sensitivity. The same effect can be arrived at by subdividing the first portion of water.

It remains to define strictly which colloid has to be selected as the most satisfactory one. A colloidal reagent has to fulfill three requirements: sensitivity, appearance, and curve. These qualities have a certain relationship with each other. The mixing speed (or the amount of the first portion of water) influences density and sensitivity in the same direction, while an increase in the concentration of mastic increases the density but lowers the sensitivity. On the other hand, the resin concentration has an influence on the shape of the curve. Consequently, no factor can be considered by itself alone; it must harmonize with the others.

As for the density, the colloidal reagent should be only slightly opalescent. It should become intensely opaque in reaction, but milky appearance is not desirable as it would diminish the visibility of precipitates. The shape of the curve is correct if its peak, on the average, corresponds with the dilution 1:8 of spinal fluid, i.e., the third tube. We determine this point by testing with several "weak" syphilitic spinal fluids.

To define the correct sensitivity, a theoretical conception must be outlined. Between the lipid tests (mainly of the serum) and the colloid tests (of the spinal fluid) there is a great resemblance in the colloidal fundamentals and (omitting the Wassermann reaction) also in the visible happening. In both types of test the characteristic effect can be brought about also by nonsyphilitic sera and spinal fluids and, with "too sensitive" reagents, even by normal sera and spinal fluids. The assumption has been made, therefore, that the lipid reactions, too, are of merely colloidal character, with only quantitative difference between the syphilitic and the normal. But the colloidal conception is not sufficient to explain fully the positive results in the lipid tests. Certain physical conditions, it is true, influence the reactivity of the reagents to syphilitic and nonsyphilitic sera in the same direction and to the same degree. But serology has learned to prepare antigens which have an increased specific sensitivity, without considerable in-

\*The selected colloid need not be the most sensitive one which corresponds with the colloidal optimum.



is another reason why diseases with only a slight change in the spinal fluid producing a weak curve, have a less distinct demarcation from syphilis than those with more marked spinal fluid changes.

The normal spinal fluid, too, has the faculty of producing a change of the syphilitic type in the colloidal reagent. This can easily be demonstrated by using a reagent with increased reactivity. However, there is one point which distinguishes the fact from similar experiences with the lipid tests: the mechanism of syphilitic and normal curves is identical, for in both instances the curve is a product of a combined action of albumin and globulin (Lange<sup>1, 9</sup>). An increased effect of globulin alone causes a spinal fluid to produce a curve of the syphilitic type.\* This occurs not only if the amount of globulin is augmented by syphilis or by another disease, but also if the globulin amount is normal and the reagent more sensitive. For this reason, no definite demarcation can exist between syphilitic and other pathologic, or between syphilitic and normal, spinal fluids. A syphilitic spinal fluid and a nonsyphilitic one, in both of which the globulin alone is increased to the same level, give the same curve, and a syphilitic curve is as strong as the protein content of the spinal fluid differs from the normal condition. Only an increase in the albumin (e.g., meningitis) produces a curve that differs fundamentally by shifting to the right.<sup>11</sup> But also this curve can, in early cases, resemble a syphilitic curve. And exactly in the same degree in which the sensitivity is raised toward syphilis, it is raised toward other pathologic changes and toward normal spinal fluid. There is no safety zone between syphilitic and nonsyphilitic spinal fluids. The aim of adjusting the mastic cannot be to catch the highest possible amount of syphilis, and the lowest amount of nonsyphilis, but by means of a linear gradation to fix the sensitivity in such a way that all spinal fluids with normal protein content barely give a negative result. This is more feasible since the normal content oscillates in narrow limits only. Any change in the protein condition, regardless of its origin, will show up in the curve. The main use of the colloid tests is not so much to base the diagnosis of syphilis upon them, as to answer the question if and how far in a case of ascertained syphilis the central nervous system is involved. For this purpose we can also evaluate a slight reaction which in itself does not differentiate syphilis from another disease or from a meaningless increase of protein in the spinal fluid (during fever). But one condition must be fulfilled, that is, constancy. We repeat: there is no safety zone, no true specific quality, and the syphilitic and nonsyphilitic changes and the normal condition shade into each other.

The manner in which we, nevertheless, can make diagnostic conclusions with regard to syphilis as well as to other diseases cannot be explained here.

In adjusting the sensitivity of the reagent at a certain level one hint is advisable. Although our technique makes the colloids very constant, a continuous control is necessary. A too high sensitivity will be recognizable in a small number of specimens, for—contrasted with the lipid reactions in such a case—not only single specimens but all of them will show themselves nonspecific. But what about the reagent that is hyposensitive? We do not always have

\*Although the merely quantitative character of the characteristic changes in the proteins is not definitely proved, this theory is supported by numerous investigations, among which the publications by Kafka and Samson are especially important.<sup>5, 20</sup>

at our disposal a spinal fluid with a tested reactivity; moreover, we will not omit the possibility given in the colloid test of having the methods at the same level in all laboratories. By adjusting the colloids in a way that all negative spinal fluids give negative results, we are not protected against hyposensitivity. It is, in my opinion, favorable to have a reagent with which the negative spinal fluid gives just a recognizable curve. The slightest degree of change indicates the standard sensitivity, and gives us an excellent control of the constancy which is the purport of my method.

In the Serologic Department of the General Hospital in Vienna, the technique described was routine for fourteen years, and was performed by me in about 70,000 specimens of spinal fluid. The most important observations were those on numerous patients who had undergone malaria treatment in the Wagner-Jauregg Clinic, Vienna, and in cooperation with Dr. B. Dattner, were kept under control for years. By its considerable constancy the standardized mastic test was able to express slightest clinical changes or to foretell a clinical development which much later became manifest. The technique was successful with resin available in this country.

#### SUMMARY

Besides sensitivity and specificity, constancy is an indispensable quality of a serologic test. In the technique of the mastic test described here, the gradation of the sensitivity is done by adding water to mastic solution in two steps, whereby the amount of the first addition is decisive for the final colloid. In this way, the sensitivity can easily be varied, and the selected reagent can repeatedly be prepared in a constant manner. Some fundamental details are discussed which add to the desired qualities of a colloidal diagnostic reagent. The technique has been useful in about 70,000 specimens of spinal fluid.

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## A SIMPLE DEVELOPING TANK FOR ELECTROCARDIOGRAMS

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TO SIMPLIFY the developing of electrocardiograms for the practitioner who does not do x-ray work, I have devised a compact three compartment tank, which makes the process easy and convenient.

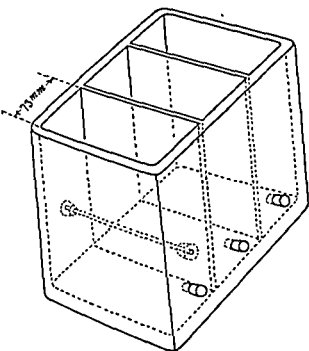


Fig. 1.

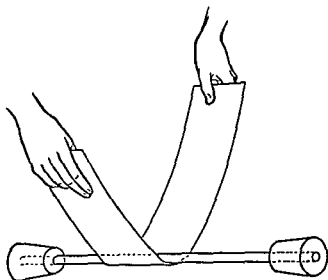


Fig. 2.

An ordinary storage battery, thoroughly cleaned out, presents three excellent chambers, perfectly arranged for the purpose (Fig. 1). The width of each chamber being about 73 mm., any electrocardiographic paper on the market today can be accommodated. The first compartment contains the developing solution. The section is so arranged that the graph may be rolled (emulsion side out) beneath a glass rod, supported at either end by one-holed No. 4 rubber stoppers, as in Fig. 2. The rod is placed about 5 cm. from the bottom, and its location may be adjusted, depending upon the amount of solution in the tank. The rubber stoppers prevent the rod from moving while the paper is rolled to get uniform developing. The middle compartment contains the water, and the third compartment, the fixing solution.

Each compartment possesses a 1 cm. bore hole at the base, about 25 mm. from the bottom, plugged with a No. 00 rubber stopper. This allows for individual emptying and cleaning of a chamber. A cover to fit the tank may be made of wood to keep the solution from outside contamination.

By the use of this tank it is possible to have all solutions within reach at any time. Solutions may be used repeatedly, space is conserved, and uniform development is obtained. It may also be used for ordinary photographic work.

# A COMPARISON OF CEDAR OIL AND OTHER MATERIALS IN THE MAKING OF SLIDES OF ATMOSPHERIC POLLEN\*

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FOR the purposes of determining the amount and kind of pollen in the air, Scheppegegrell<sup>1</sup> in 1917 exposed plates prepared by applying glycerin to the central square inch of an ordinary microscope slide which had previously been cleaned with alcohol. Various preparations were tried at that time, but glycerin was found to be superior to any other. When the air was extremely moist, the glycerin deliquesced rapidly, but this deliquescence was overcome by substituting boiled linseed oil. In the beginning of his work, Scheppegegrell attempted to expose slides at right angles to the direction of the wind; later he found this to be impractical due to the limited range of a single slide. He found the most practical method to be the simple exposure of the slides to the wind without the use of any special apparatus. After the slides had been exposed for twenty-four hours, a drop of Lugol's solution was placed on the glycerin surface, thus facilitating the counting. The grass pollens were stained blue black in contradistinction to the other pollens, which were usually stained brown.

In 1924 Scheppegegrell<sup>2</sup> made pollen counts from slides carried in an airplane. These slides were smeared with either glycerin or vaseline. The combination of glycerin and Lugol's solution facilitated the counting, but the preparation was rapidly dried by the great velocity of the wind to which the plates were exposed. This was not the case with the slides on which the vaseline film was employed.

Koessler and Durham<sup>3</sup> exposed slides in various parts of Chicago in 1926. They used corn oil and found it to be more satisfactory than glycerin because it did not absorb moisture and thus distort the pollen grains. They found that stains were unnecessary in their study.

In his study of the atmospheric pollen and botanic flora of the East Shore of San Francisco Bay, Rowe<sup>4</sup> used slides covered with a film of vaseline. The slides were exposed in an upright position, with the vaseline facing the prevailing wind, for a period of twenty-four hours. He found that the pollen grains adhered to the slides covered with white vaseline and retained their dry, shrunken shapes.

Detweiler and Hurst<sup>5</sup> used slides coated with a thin layer of glycerin jelly. The jelly was prepared according to Kaiser's formula and subsequently remelted in a water bath. It was then smeared over the slide with a glass rod. an area of the slide equivalent to the surface of a cover slip being prepared.

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These slides were exposed on window ledges in various parts of Toronto for periods of twenty-four hours. When the slides were collected, a drop of glycerin jelly, protected by a cover slip, was added.

Wodehouse<sup>6</sup> described various stains used in combination with a glycerin jelly. The methyl green stain proved to be the most satisfactory of the staining preparations.

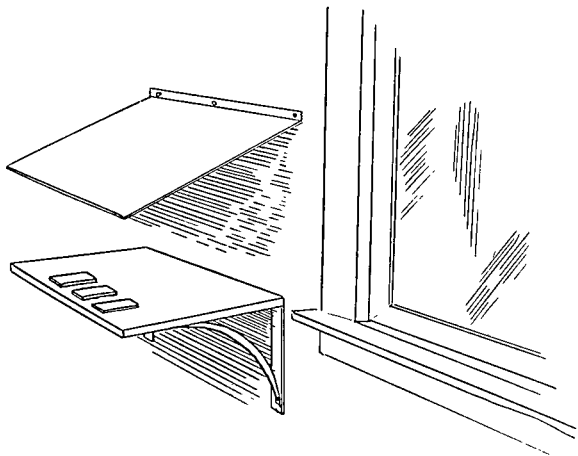


Fig. 1.—Shelf with cover, such as was used for exposure of slides to atmospheric pollen.

I encountered certain difficulties at the Vaughan-Graham Clinic, in Richmond, Va., in pollen counts made on a group of slides from the Binghamton, N. Y., area. A number of the slides had dried out, others had been exposed in the rain, and still others, due to the position in which they had been placed, released an excessive amount of mineral oil on one or the other side.

I then became interested in developing a method that would avoid these conditions and would, in addition, facilitate the identification of the pollen grains. Consequently, I tried a number of materials, including mineral oil, glycerin, white vaseline, corn oil, almond oil, glycerin jelly, and cedar oil.

When vaseline was employed, it was smeared over the middle third of the slides and melted slowly over a Bunsen flame in order that a smooth surface might be obtained. The oils were applied to the slides without the aid of heat.

The cedar oil was that used with the oil-immersion lens. Some of it was found to be rather thick at times, and the addition of several drops of xylene was necessary to thin it. The oil was applied to the slide (which had previously been cleaned with alcohol) with the end of a glass rod, using only a small drop for each preparation. The rod was then moved horizontally on the slide

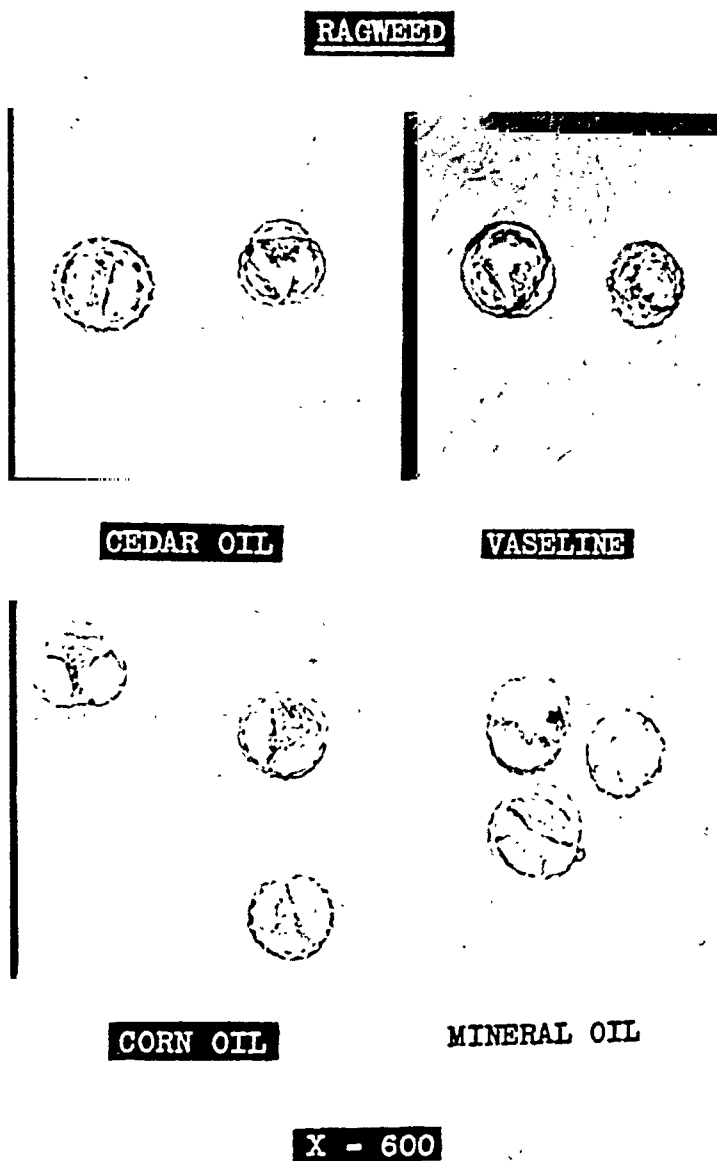


Fig. 2.—Photomicrographs of ragweed pollen grains showing comparison of cedar oil, corn oil, vaseline, and mineral oil.

to facilitate an even distribution over the middle third. Slides which we would probably want to file and store we protected with a cover slip. The slides were placed side by side on a shelf that extended about 18 inches beyond a window ledge. This shelf was protected from the rain by a metal cover suspended about 15 inches above, where it was attached to the side of the building (Fig. 1). The slides were observed before and after the mounting. Those for which cover slips had been used were found to be more satisfactory for study. All slides with the oil preparations presented a sharper distinction of the pollen morphology than did those with vaseline and plain glycerin jelly (Figs. 2 and 3).



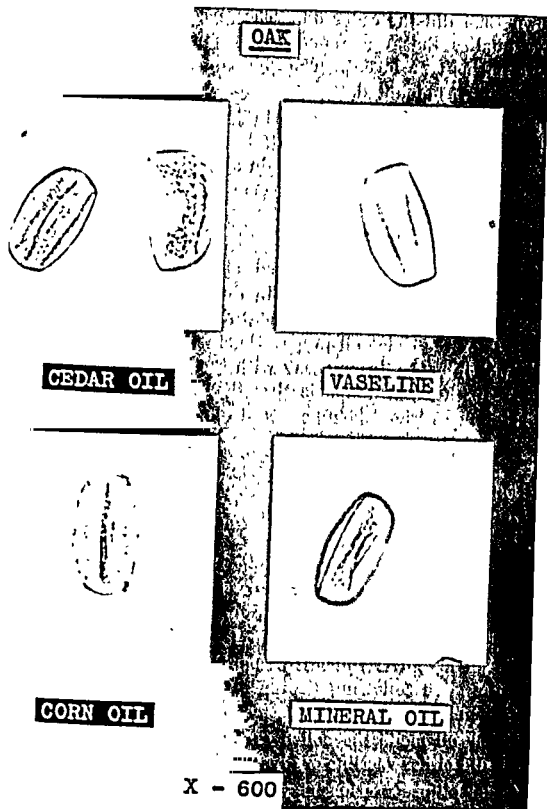


Fig. 3.—Photomicrographs of oak pollen grains showing comparison of cedar oil, corn oil, vaseline, and mineral oil.

The cedar oil preparations were found to be the most satisfactory from all points of view. As compared with vaseline, the cedar oil made the pollen grains more translucent on microscopic examination, while an amorphous or crystalline area was apparent in the slides prepared with vaseline, due to its cooling in preparation. After the cedar oil slides were exposed for a period of twenty-four hours, the oil would at times set (if the weather was very warm). From the beginning of the exposure it retained its adhesive qualities, which were more pronounced than the other oils.

All the other oils were found to be rather thin and tended to extend beyond the area over which they were applied. Later, when they were placed in a dust-proof slide box, they settled more readily to the side of the slide that

was down than did the cedar oil. The glycerin jelly preparations lacked the adhesive quality that was found in the slides with thin films of cedar oil and were not as translucent. The glycerin jelly was heated each time before the cover slip was mounted.

Plain glycerin jelly and glycerin jelly containing various stains in different concentrations were studied, but none of the stained preparations were found to be superior to the plain glycerin or as good as the oil preparations for routine work; however, some of the stained preparations showed more detailed structure.

Both sheltered and unsheltered slides were exposed at the same time, but there was little difference in the total pollen counts.

Cedar oil slides may be filed in dust-proof slide boxes following their exposure to atmospheric pollen without any other preparation; however, I did mount some of the cedar oil preparations with Canada balsam and cover slip. This did not interfere in any way with the distribution of the pollens on the slides. A further advantage is that they may be kept in this manner for future reference without being exposed to contamination with dust.

#### SUMMARY AND CONCLUSIONS

1. Comparative studies were made of atmospheric pollen slides prepared with glycerin jelly, glycerin, vaseline, corn oil, almond oil, mineral oil, and cedar oil.

2. Glycerin jelly containing stains presented an advantage over plain glycerin jelly where study of the structural detail was desired, but not for routine use.

3. Cedar oil was found to be preferable to the other oils in that it remained in the area over which it was applied and did not spread or run off the sides of the slide following exposure to atmospheric pollen. Cedar oil appeared to be more adhesive than some of the other preparations.

4. Cedar oil made the pollen grains more translucent to the microscope light than did either vaseline or glycerin jelly.

5. Cedar oil preparations may be filed, either mounted with Canada balsam and cover slip or unmounted, in dust-proof slide boxes.

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## THE VENTILOGRAPH: AN IMPROVED RECORDING VENTILOMETER AND ITS APPLICATIONS

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TWO years ago one of us described a recording ventilometer<sup>1</sup> which, as an auxiliary unit attached to the familiar Benedict-Roth metabolism apparatus, enables recording on the kymograph chart, in addition to the customary spirogram, a second curve which by analogy is termed the *ventilogram*. The spirogram by its slope denotes the amount of oxygen absorbed by the patient per unit of time. The ventilogram serves as an indication and measure of the rate and amount of pulmonary ventilation during the corresponding period.

Since the publication of the first paper, the ventilograph, as this new unit has been termed, has been used experimentally in several research centers. Based on the experience thus accumulated, a new and improved model has been developed. It is the purpose of this paper to describe the improvements and to comment on the various uses to which this apparatus may be put.

The ventilograph consists of a mechanism that actuates a recording pen moving parallel to the pen which records the spirogram. The ventilograph pen is elevated one-twenty-fifth of the distance the spirographic pen moves during inspiration only. Since oxygen consumption is volumetrically about one-twenty-fifth of the pulmonary ventilation, the spirogram and ventilogram tend to follow substantially parallel courses, as shown in Fig. 2.

An assembled view of the mechanism of the ventilograph head is shown in Fig. 1. *f* is the pulley support arm of the standard Benedict-Roth metabolism apparatus. *A* is the original pulley wheel, and *a-a* is the original pulley chain, one end of which is attached to the oxygen bell and the other to the spirographic pen. This wheel *A* moves back and forth as the bell rises and falls. To the original fork *f* is added a support for the gear wheels, and an extended shaft for the second pulley *B*. Within the gear case *G* is a beaded hard fiber wheel which, through the action of two blade-type pawls, is permitted to move in one direction only. One pawl is attached to the pulley wheel *A* and advances the fiber wheel during the inspiratory excursion of the bell. A second pawl attached to the frame holds the fiber wheel stationary during the reverse excursion of *A*. The unidirectional movements of this fiber wheel are communicated through a train of reduction gears to the second pulley wheel *B*, which has the same diameter as *A*. Attached to the periphery of *B* by the set screw *s* is a chain *b*; the rotation of *B* in the direction of the arrow thus serves to elevate the pen of the ventilograph which is attached to the lower end of chain *b*. This pen records

the ventilogram, a line which by its slope denotes the sum total of the inspiratory excursions of the bell on a  $1/25$  scale. A safety device is incorporated in the gear train to prevent overwinding. The ventilograph pen may be reset to any desired position by pulling sleeve *r* outward to disengage the gears.

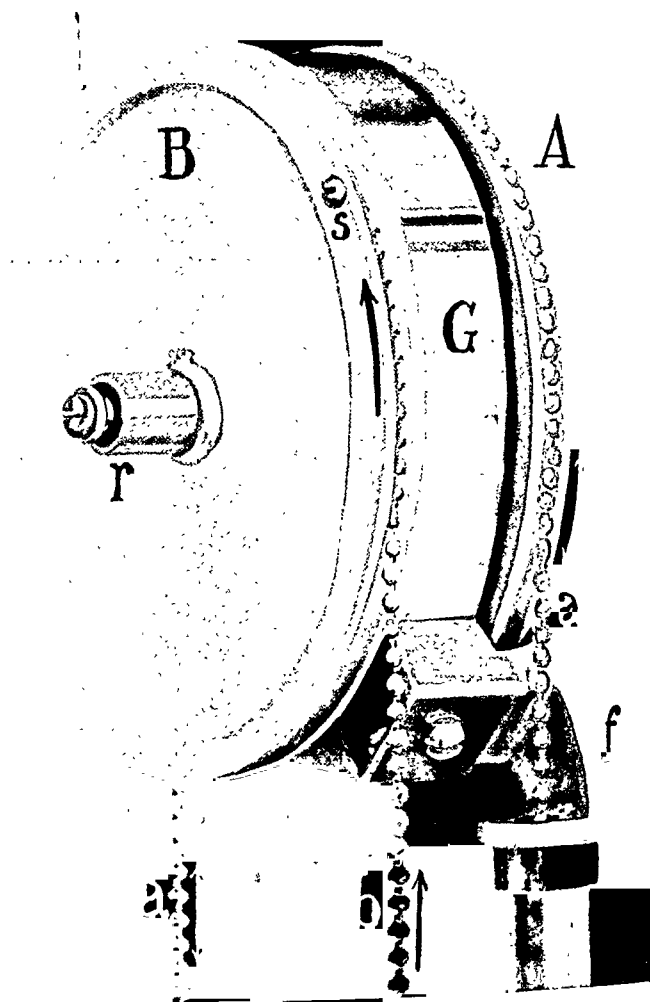


Fig. 1.—The pulley wheel assembly of the ventilogram. *f* is the fork of the stock model basal metabolimeter, supporting *A* the original pulley wheel of the machine. Chain *a* runs on the periphery of this wheel and makes reciprocal excursions with the oxygen bell, recording the familiar spiogram of oxygen consumption. A set of gears within the case *G* activates the added wheel *B* so that it moves in the inspiratory direction only, raising the chain *b* fixed at the screw *s* at one-twenty-fifth the amount of the inspiratory phases of *A*. *r* is a manual reset mechanism.

In the first model of the ventilograph, the unidirectional wheel was of bakelite and had a knurled periphery upon which two pawls bore at an angle, the combination acting as a ratchet in the ordinary sense. Although the wheel was given a very fine knurling (0.02 inch), it was discovered that when respiratory excursions were small, the error introduced by the failure of the pawls always to drop exactly into one of the spaces in the knurling might amount to as much as 5 per cent, though ordinarily it did not exceed 2 per cent with human beings.

In the present model the ratchet mechanism was modified by giving the wheel two peripheral beads, with a modified pawl resting on each. By proper choice of the wheel material, and by selecting a pawl blade of appropriate springiness, sharpened and mounted at a certain angle, we found that the device acted as a true ratchet, although there are no "teeth" on the periphery of the wheel. Provision has also been made for the adjustment of the tension of the pawl blades against the wheel by means of conveniently accessible screws.

By actual measurement the loss has been reduced to a point below the error of available checking means, substantially less than 1 per cent. In addition, two further advantages have been gained. Through the elimination of the ratchet teeth, the device has been made silent in operation, and since the pawl blades are more flexible, and do not have to lift over a series of teeth, the friction introduced, though scarcely appreciable before, has been reduced to one-third or one-fourth of its former value.

The ventilograph has proved a great timesaver wherever the determination of pulmonary ventilation has been desired in the course of spirometric experiments using closed circuit metabolism apparatus as such or in modified form. With such apparatus, the only previous methods of estimation were the measurement of the cumulative length of successive inspiration lines on the kymographic record, either by calipers or by a map tracer. Both methods are fatiguing, time-consuming, and subject to considerable possible error.

A further advantage of the ventilograph is that the slope of the ventilogram may be used, even for periods of less than a minute, as an index of the rate of ventilation, as distinguished from the *amount* of ventilation over a longer period. It is probably the only satisfactory means of estimating ventilation rate for short periods, for while the general appearance of the spirogram (i.e., the depth of respiration and the rate) furnishes a rough indication, the difficulty of mentally integrating both those factors is obvious, as is its measurement from the spirogram as previously described.

The use of the ventilograph to determine ventilation rates is illustrated in Fig. 2. The graph is recorded from right to left. Upstrokes of the spirogram indicate inhalation; downstrokes, exhalation. A given point on the ventilogram corresponds to a point on the spirogram one space to the left, since the pens record that distance apart. Vertical lines represent minutes.

To determine the ventilation rate for a given portion of the ventilogram, a line is drawn paralleling that portion in the same manner as in oxygen determination from the spirogram. This line may be as long as necessary to enable accurate measurements of its rise in a given number of minutes. From the figure thus obtained, the rise per minute is calculated and multiplied by 0.52, which factor yields the ventilation rate in terms of liters per minute.

For instance, line *AA'* represents the initial slope of the ventilogram, during the period of quiet breathing indicated on the spirogram. The line rises 50 mm. in four minutes, or 12.5 mm. per minute. Multiplying 12.5 by 0.52 gives the ventilation rate, 6.50 liters per minute. Similarly, line *BB'* represents an in-

creased ventilation rate produced by more rapid breathing during a one-half minute period. The line rises 56 mm. in two minutes, or 23 mm. per minute. Similarly, line  $CC'$  represents a still higher ventilation rate of 25.45 liters per minute.

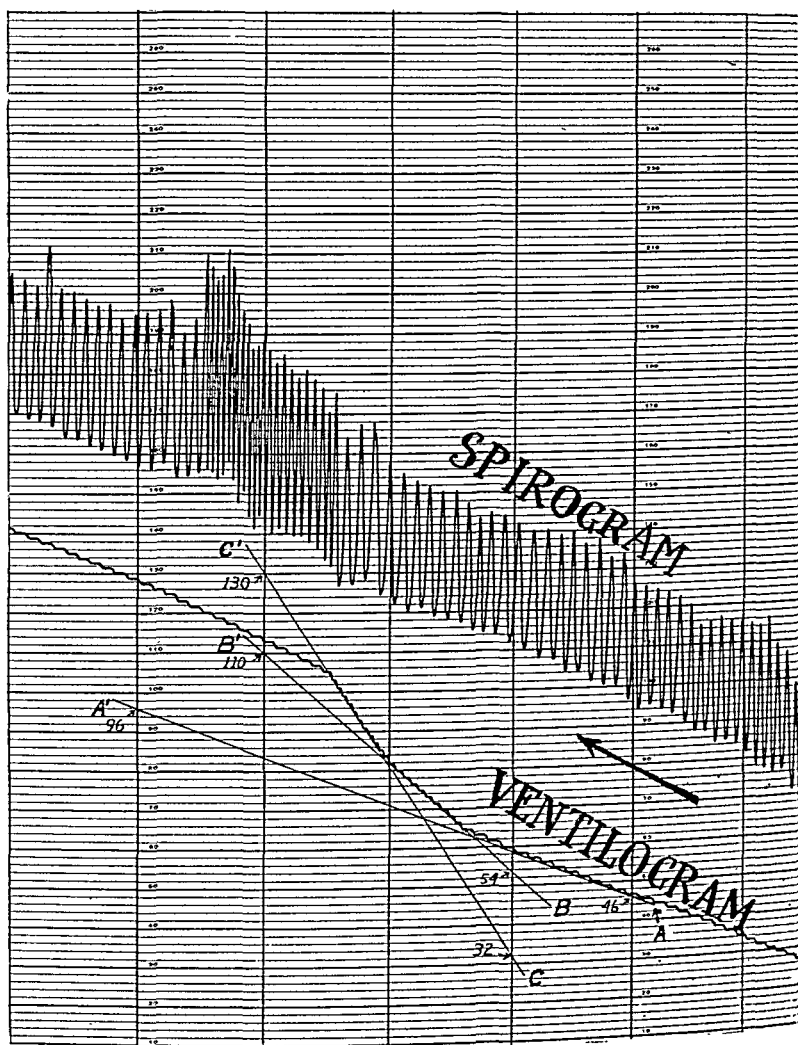


Fig. 2.—The spirogram is recorded by the pen attached to the spirometer bell by a chain passing over the original reciprocal pulley (Fig. 1A). The ventilogram is recorded by a pen attached to chain  $b$  (Fig. 1). This is raised by pulley  $B$  through a distance equaling one-twenty-fifth the total inspiratory excursions of the spirogram. The spirogram gives both total ventilation and, by extension of sections of the curve, ventilation rates for selected periods. Since the pens cannot be superimposed, any point on the spirogram has a known constant time lag on the ventilogram curve—in this case exactly one minute.

The ventilograph was first designed to facilitate the investigation of the "oxygen-absorbing power" index proposed by Ziegler<sup>2</sup> and the similar "ventilation equivalent for oxygen" index proposed by Monerief.<sup>3</sup> Since then it has found other applications. Most of these are as yet in the research field, but experience makes it likely that clinical apparatus embodying the ventilograph will shortly be available.

Cournand and Richards<sup>4</sup> have developed an apparatus of a modified Benedict-Roth type, with ventilograph and other accessory equipment, for testing respiratory functional efficiency in chest diseases and other conditions.

Barach has used ventilograph-equipped spirometers in research on various phases of the diagnosis and treatment of asthma and emphysema,<sup>5</sup> in the study of the effect of oxygen and helium therapy on respiration<sup>6</sup> and in a field of research as yet relatively untouched in this country, bronchoscopic spirometry or "bronchospirometry," in which the air from the right and left lungs is separately conducted through a special bronchoscope to individual spirometers.<sup>7</sup> Pinner<sup>8a</sup> is also using similar equipment for bronchospirometry in clinical practice.

Recent work by Landt and Benjamin<sup>8</sup> indicates the possibility of studying cardiac functional capacity through the response of pulmonary ventilation to progressive lowering of the oxygen content of the inspired air. The ventilograph is being used in further research in this field. It is possible that similar application may be made in apparatus for the "metabolic exercise tolerance test" for patients with cardiac disease, proposed by Soskin and co-workers.<sup>9</sup>

Work is currently in progress at the Pierce Laboratory for Hygiene at Yale University, for the determination of the physiologic effects (including effects on metabolism and respiration) of various conditions of temperature, humidity, and radiant energy in the environment.<sup>10</sup> Here again a ventilograph-equipped metabolism apparatus is an essential part of the equipment. Similar apparatus is in use at the Aero-Medical Research Laboratory at Wright Field, where it is applied in some phases of aviation respiration research, not yet completed.<sup>11</sup>

Since the publication of the work of Nielsen and Roth<sup>12</sup> on the significance of various types of spiograms, little has been published on the subject of clinical spirometry. A recent renewal of interest has occurred, particularly in the field of clinical psychiatry, and it appears likely that with the availability of a convenient means of studying the pulmonary ventilation rate, this factor will be considered as an adjunct to the appraisal of the spiogram proper. Investigation along this line is currently being initiated at the Massachusetts General Hospital in Boston<sup>13</sup> and the Presbyterian Hospital in New York.

#### SUMMARY

A new and improved model of the ventilograph, a means of graphically recording the pulmonary ventilation with closed-circuit recording spirometers of the Benedict-Roth type, is described, and its applications in the field of research and clinical medicine are discussed.

The ventilograph may be obtained on special order from the makers, Warren E. Collins, Inc., 555 Huntington Avenue, Boston, Mass.

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## AN APPARATUS FOR THE MANUFACTURE OF WAX PARAFFIN AMPOULES FOR SILVER NITRATE SOLUTION USED IN THE PREVENTION OF OPHTHALMIA NEONATORUM\*

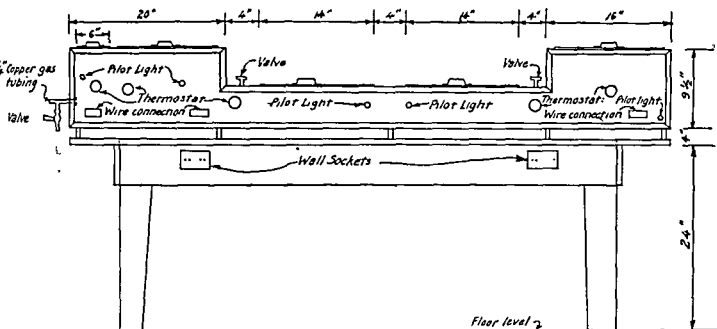
DEWEY M. WELLS, MONTGOMERY, ALA.

THE preparation and distribution of silver nitrate solution for the prevention of ophthalmia neonatorum has become a routine procedure of many public health laboratories. A method for the manufacture of ampoules composed of a mixture of beeswax, paraffin, and paraffin oil, for the distribution of this product, was originally described by Young.<sup>1</sup> Silver nitrate solution stored in these ampoules often deteriorated rather rapidly, resulting in the formation of a black precipitate and a strongly acid solution. Bunney<sup>2</sup> showed that the beeswax was responsible for this deterioration, and solved the problem by making a paraffin ampoule and coating it with beeswax. In such ampoules the silver nitrate solution never comes in contact with the beeswax—which is essential to prevent cracking or crumbling—and, therefore, is not subject to the deleterious effects of this material.

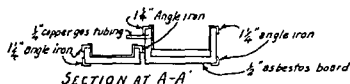
Bunney's method consists essentially of first dipping brass spindles twice in a melted mixture of 12 per cent paraffin oil and 88 per cent paraffin of 68° to 72° C. melting point. The spindles are then dipped several times in a melted mixture containing 71 per cent beeswax, 8 per cent paraffin oil, and 21 per cent paraffin of 56° C. melting point. The ampoules are filled with silver nitrate solution and sealed by running melted paraffin into them on top of the silver nitrate solution.

\*From the Bureau of Laboratories, State Department of Public Health, Montgomery.  
Received for publication, October 7, 1939.

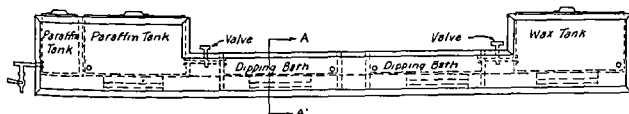




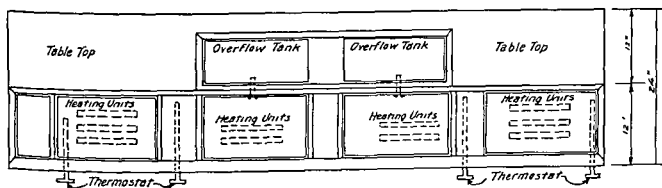
FRONT ELEVATION



SECTION AT A-A'



CONSTRUCTION DETAILS—FRONT ELEVATION



CONSTRUCTION DETAILS—TOP VIEW

Fig. 1.—Paraffin wax bath.

An apparatus designed to facilitate manufacture of paraffin-lined beeswax ampoules of the type described by Bunney is outlined here. These ampoules have been made in this laboratory for two years, and the following problems were encountered in the use of the old type of apparatus:

1. It was necessary to have three melting baths; one for the 68° to 72° C. melting point paraffin; one for the beeswax-paraffin mixture; and one for the paraffin used for sealing.
2. Maintenance of the beeswax mixture and the high melting point paraffin at a constant depth in the dipping tank.
3. Proper control of the temperature of the two tanks of paraffin and the beeswax at their respective melting points so that ampoules of uniform thickness could be made.

4. Arrangement of the different units so that the whole process could be carried on with a minimum number of workers.

The apparatus shown in Fig. 1 was designed to overcome these difficulties in manufacture. The unit<sup>\*</sup> consists of three melting tanks, two dipping vats, and two overflow pans, each of which is equipped with separate heating units. The three melting tanks and the two dipping vats are each equipped with separate thermostats, so that each can be kept at the exact temperature desired. Each vat has an overflow pipe located in the side, and at such distance from the bottom that the beeswax and paraffin can be maintained at a constant level, thus assuring ampoules of uniform length. Excess wax or paraffin flowing from the melting tank to the dipping vat runs through the overflow pipes into the pans, where it can be kept melted and returned at intervals to the melting tank.

On the end of the assembly is located the melting tank for paraffin used for sealing the filled ampoules. The melted paraffin is dispensed through the hand-operated metal valve at the end of the tank. The apparatus is assembled in a framework of angle iron and asbestos board, mounted on a table of suitable dimensions and height. The tanks, covers, and finishings are of stainless steel. Most of the electrical wiring is concealed underneath the table top with short wires equipped with detachable connections coming from the sides of the table for attaching to the separate heating units. Electric current is provided by a single long extension cord that may be attached to any light socket or wall plug. The legs of the table are equipped with casters so that the whole unit can be moved easily for storage when not in use.

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\*This apparatus was built by the Apparatus and Specialty Co., Lansing, Mich., for the Bureau of Laboratories of the Alabama State Department of Health.

## A SIMPLE BLOWFLY CAGE FOR THE CULTURE OF SURGICAL MAGGOTS\*

MAURICE S. TARSHIS, B.A., M.S., CORVALLIS, ORE.

IN A PREVIOUS article in this JOURNAL<sup>1</sup> a simple blowfly cage for the culture of surgical maggots was described. Since then another type of cage has been devised. This cage, which has proved very satisfactory, is much simpler in design and costs less to construct. It is hoped that it will prove useful not only to those interested in the culture of surgical maggots, but also to those interested in the rearing of the blowfly or of other flies for experimental studies.

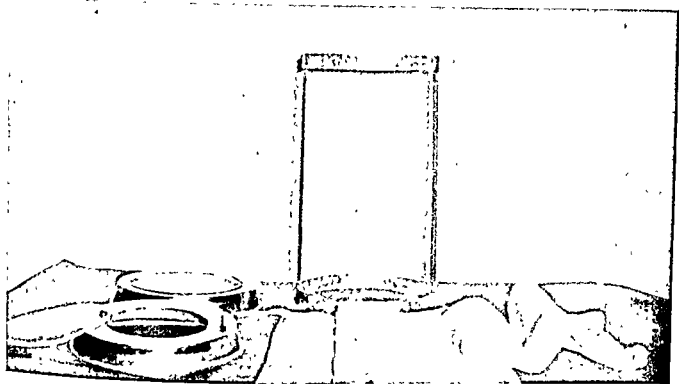


Fig. 1.—Framework and other parts of cage. Note construction of various parts.

The frame of the cage consists of two galvanized iron rims, 1 inch wide and 10 inches in diameter, connected by parallel wooden supports, 16 inches high and 1 inch wide (Fig. 1). These wooden supports are fastened to the inside of the iron rims by means of flat-headed bolts and nuts. If greater rigidity is desired, the number of wooden supports may be increased; however, two supports have been found to be sufficient. The edges of the iron rims are coated with liquid solder in order to facilitate placing the cloth cover on the cage as well as to obviate tearing of the cloth by jagged edges.

\*From the Department of Zoology, Oregon State College, Corvallis.  
Received for publication, October 7, 1939.



Fig. 2.—Showing arrangement of cloth cover. Note paper clips at top of cage and sleeve for entry into cage.

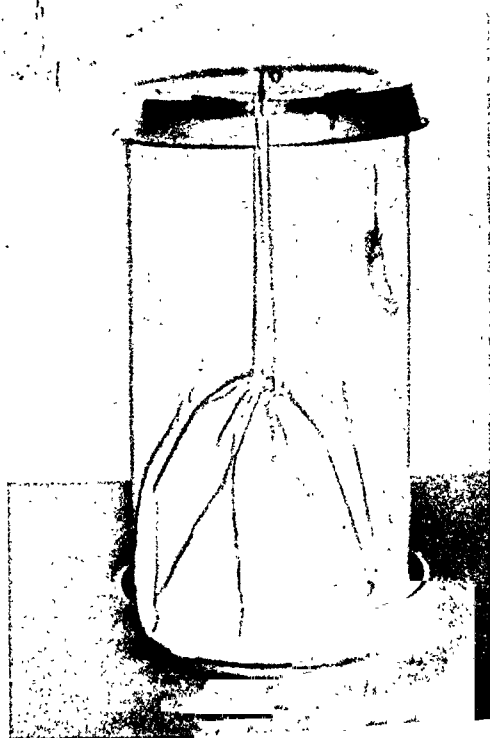


Fig. 3.—Complete arrangement of cage. Paper tag near top of cage is used for record. The tag is attached to the cloth cover by means of a small piece of flexible wire.

The cover of the cage consists of muslin sheeting. It is made to fit snugly around the frame. The cloth cover is allowed to extend for about an inch beyond the top and the bottom of the cage. To fasten the cover to the cage, the 1 inch border at each end of the cage is folded over each iron rim and held in place by means of paper clips (Fig. 2). A sleeve, 12 inches long and 6 inches in diameter, is sewed into this cover several inches from the bottom. A  $\frac{1}{4}$  inch hem is made at the free end of the sleeve, with two small openings at opposite sides. Through this hem are drawn two strings, in the same manner as the draw strings of a purse (Fig. 2). The ends of each draw string are tied, and when the sleeve is not in use, the strings are attached to a small inverted bolt near the edge of the top of the cage. A nut holds this bolt in place (Fig. 3). The sleeve not only facilitates entry into the cage, but also prevents the flies from escaping during the placement and removal of food. A zipper opening makes it easy to arrange and remove the cloth cover. When the zipper is used, it is not necessary to make the sleeve, for by partly opening the zipper, entrance into the cage can be made satisfactorily. When a zipper is sewed into the cover, it should be placed so that it opens from the bottom to the top of the cage. In the placement and removal of food, however, the opposite side of the cage is illuminated near the top by means of a desk lamp. The light attracts the flies and obviates the danger of their escaping when the zipper is opened.

The cage is placed in a 10 inch metal pan, which serves as the bottom, and is covered by another 10 inch metal pan (Fig. 3). In each pan is cut a circular opening, about 8 inches in diameter, and each opening is covered with a 9 inch, transparent, celluloid disk. The celluloid disk for the pan to be used on the bottom of the cage is fastened to the pan with four small, flat-headed bolts and nuts. The disk for the pan to be used on the top of the cage is held in place with only one small, loosely screwed bolt and nut, so that it can be swung in and out of place. This arrangement permits the transfer of flies from soiled to clean cages.

To transfer the flies the clean cage is inverted over the soiled one, the loose celluloid disk on each cage is swung outward, and the bottom of the inverted cage is illuminated. The flies, being positively phototropic, are quickly attracted to the illuminated surface. When the flies have migrated to the clean cage, the celluloid disks are moved back in place, and the inverted cage is placed upright. The transparent disk also permits observation into the cage. This is especially convenient during the placement and removal of food dishes.

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## A METHOD FOR THE DETERMINATION OF SUGAR IN SMALL AMOUNTS (0.02 C.C.) OF BLOOD\*

NIELS C. KLENDSHOJ, M.D., AND ROGER S. HUBBARD, PH.D.  
BUFFALO, N. Y.

DETERMINATIONS of the concentration of sugar in the blood are most conveniently made upon samples drawn from a vein. Such samples are usually so large that the analyst can make measurements with a fairly high degree of accuracy, i.e., 2 c.c. are measured with an accuracy of between 0.5 and 1 per cent. In a fair number of patients, particularly children, it is sometimes difficult, or practically impossible, to obtain this amount of material by a satisfactory venipuncture. To meet these difficulties a number of micro-methods of analysis have been developed and adapted to the study of blood from the capillaries. In all methods of this kind there are inherent technical disadvantages. When a gravimetric method of measuring the sample<sup>1</sup> is employed, the cost of equipment and the special precautions necessary in the preliminary treatment make routine use quite unsatisfactory. In methods using an initial volumetric measurement of which several have been described,<sup>2, 3</sup> the decrease in accuracy of measuring the sample serves as one source of difficulty. This seems unavoidable, for the measurement of 0.1 c.c. with an accuracy of 1 per cent can be made only when great precautions in handling the material can be observed, and such precautions cannot be taken when working with blood oozing from the finger or ear. In our experience another difficulty was encountered when an attempt was made to apply these methods to the study of patients. Not infrequently it has been found almost impossible to obtain a suitable 0.1 c.c. sample from the usual puncture wound if the clinician or technician has not acquired considerable practice in the manipulations involved. It seemed to us that it might be worth while to investigate the effect of decreasing the size of the sample taken for analysis, and, even at the expense of an increased inaccuracy of the initial measurement, to decrease the amount of technical skill necessary for obtaining the material. Since analyses of 0.02 c.c. of blood obtained from a superficial puncture of the finger or ear are commonly made in the determination of hemoglobin, and since many, if not all medical students, interns, and technicians are thoroughly drilled in the technique of making such estimations, an attempt was made to see whether this amount of blood would serve for a fairly satisfactory determination of the blood sugar concentration.

\*From the Laboratory of the Buffalo General Hospital and the Department of Pharmacology, University of Buffalo, School of Medicine, Buffalo.  
Received for publication, October 13, 1939.

Since many clinical laboratories have found that colorimetric methods are satisfactory for diagnostic purposes, attention was centered upon developing a colorimetric procedure. Folin and Malmros<sup>1</sup> have described a modification of the method of Hagedorn and Jensen<sup>2</sup> in which 0.1 c.c. of blood from the finger is used, and the final estimation is based upon the determination of the amount of Prussian blue formed under standard conditions. The intensity of the final color obtained in this procedure, and the relative stability of the oxidation-reduction system employed in it, made it seem probable that relatively slight modifications in the technique would make it possible to analyze even smaller amounts of blood in a similar way. A study was, therefore, undertaken, and the method described below, which we believe is adapted to the study of finger blood for diagnostic purposes, was developed.

*Apparatus.*—(1) A pipette of fine capillary tubing, graduated at 0.02 c.c. The Sahli pipette for the estimation of hemoglobin was used for this purpose.

(2) A microcolorimeter, capable of measuring the amount of color in 1 or 2 c.c. of solution.

(3) Small tubes, approximately 3 inches in length and 0.5 inch in diameter, such as are used for serologic tests. A water bath, standard pipettes, and flasks, such as are used in routine work complete the necessary equipment.

*Reagents.*—(1) Dilute tungstic acid. Measure 5 c.c. of 10 per cent sodium tungstate into a 250 c.c. volumetric flask. Add water to give a volume of about 200 c.c. Add, while shaking, 5 c.c. of  $2/3$  N sulfuric acid, and dilute to 250 c.c. with distilled water. This reagent, which is the one recommended by Folin and Malmros for the precipitation of protein, must be prepared freshly every two weeks. It should contain no measurable amount of reducing material, i.e., it should give no color when a blank analysis is carried through the procedure.

(2) Potassium ferrieyanide. Dissolve 0.05 Gm. of pure potassium ferrieyanide in 100 c.c. of distilled water. This reagent should be discarded when precipitate forms, or when a blue color develops upon the addition of the iron solution.

(3) Sodium carbonate. Dissolve 1.6 Gm. of anhydrous sodium carbonate in 100 c.c. of distilled water. The ferrieyanide and carbonate solutions have been somewhat modified from those described by Folin and Malmros.

(4) Ferric iron solution. Dissolve 0.10 Gm. of ferric ammonium sulfate in 50 c.c. of water. Add 5 c.c. of syrupy (85 per cent) phosphoric acid and 0.30 Gm. of "duponol."\* The solution is made to a volume of 100 c.c.

(5) Standard glucose solution. A stock solution containing 0.2 per cent of pure glucose in water is prepared. This is covered with a thin layer of toluol and kept in the refrigerator. The standard used in the determination is prepared from this stock by diluting 1 c.c. of it to 200 c.c. with distilled water. This dilute standard must be prepared fresh every day.

\*"Duponol" is the trade name for an emulsifying agent prepared and sold by E. I. DuPont de Nemours & Co. It is added to make the suspension of Prussian blue more permanent. We encountered some difficulty in preparing the gum solution recommended by Folin and Malmros for this purpose, and have, therefore, substituted "duponol" in their procedure. This preparation, in the amount used, maintained the Prussian blue in clear solution for between twenty-four and forty-eight hours.

*Method.*—Measure 2 c.c. of the tungstic acid solution into a small test tube. The blood, either capillary blood from a puncture wound or oxalated blood, is drawn exactly to the mark in a Sahli pipette, and any excess is wiped off the stem. The blood is then quickly expelled into the tungstic acid solution. It usually tends to settle to the bottom, and the clear supernatant fluid is drawn two or three times into the pipette and returned to the test tube to insure thorough rinsing. The blood and the tungstic acid are then mixed well, and the tube is allowed to stand two or three minutes or more. It is then centrifuged. One cubic centimeter of the supernatant fluid is measured into the tube described above, and 1 c.c. of the diluted standard glucose is put into a similar tube. To each is added 0.5 c.c. of potassium ferricyanide, followed by 0.2 c.c. of carbonate. Both tubes are placed in a boiling water bath for eight minutes, and then cooled in running water. One cubic centimeter of the ferric iron solution is then added to each, and the colors compared after fifteen minutes or more in a microcolorimeter.

TABLE I

COMPARISON OF RESULTS OBTAINED BY THE FOLIN-WU METHOD AND THE MICROMETHOD

FOLIN-WU METHOD	PROPOSED METHOD	DILUTION USED	DIFFERENCE PER CENT	DIFFERENCE
mg./100 c.c.	mg./100 c.c.			mg./100 c.c.
99	102	1:1	3.0	3
96	103	1:1	7.3	7
95	87	1:1	8.4	8
80	91	1:1	13.7	11
133	118	1:1	11.3	15
165	145	1:1	12.1	20
392	380	1:4	3.1	12
325	325	1:4	0.0	0
103	105	1:1	1.9	2
143	133	1:1	7.0	10
84	85	1:1	1.2	1
580	616	1:4	6.2	36
58	61	1:1	5.2	3
75	78	1:1	4.0	3
222	214	1:1	3.6	8
200	210	1:1	5.0	10
196	184	1:1	6.1	12
215	228	1:1	6.0	13
300	324	1:2	8.0	24
304	291	1:2	4.3	13
360	341	1:2	5.3	19
292	315	1:2	7.9	23
186	169	1:1	9.1	17
460	489	1:4	6.3	29
32	42	1:1	31.0	10
Mean	207.4			209.4

When the glucose concentration is 50 to 200 mg. per 100 c.c. of blood, the method can be followed as described. For concentrations less than 50 mg. the interference of the yellow color makes the reading inaccurate. When the blood sugar concentration is higher than 200 mg. per 100 c.c., the supernatant liquid must be diluted before analysis. When the concentration is between 200 and 400 mg., satisfactory results are obtained if the filtrate is diluted 1:2 or 1:4 with distilled water; blood sugar concentrations between 400 and 800 mg. require dilution of 1:4 or 1:8. If the readings are kept within this range, it is not



necessary to use a light filter to prevent significant interference by the yellow color of the unreduced potassium ferricyanide.

*Calculations.*— $\frac{S}{U} \times \text{Standard} \times 1.01 = \text{Mg. glucose per 100 c.c. of blood.}$

Where  $S$  = reading of standard,  $U$  = reading of unknown, and standard = 100 mg./100 c.c. If the filtrate was diluted 1:2 or 1:4 or 1:8, the results must be multiplied by the appropriate factor—2, 4, or 8 as the case may be. The standard is so diluted that the value is 100 when the colors of the standard and the unknown are alike, and the procedure is carried through upon the undiluted supernatant fluid as previously described. The factor 1.01 is used because the total volume of blood plus tungstic acid solution is 2.02 c.c., and the amount of blood contained in it is 0.02 c.c. A simplified expression of the formula is  $\frac{101S}{U}$ . Dilution of supernatant fluid = Mg. glucose per 100 c.c. of blood.

The method was standardized by making simultaneous determinations by the method of Folin and Wu<sup>5</sup> and by the technique described upon samples of oxalated venous blood. The results are presented in Table I, in which both the actual and the percentage differences between the two values are given. It is evident that in spite of the errors inherent in measuring such small quantities of blood, the agreement is close enough to suggest that the method should be satisfactory for diagnostic purposes. The percentage difference between the two methods has been summarized in Table II.

TABLE II

PER CENT DIFFERENCES BETWEEN THE RESULTS BY THE FOLIN-WU METHOD AND THE MICRO-METHOD

PER CENT RANGE	NUMBER
0 to 5	9 pairs of values
5.1 to 10	12 pairs of values
10.1 to 15	3 pairs of values
Over 15	1 pair of values

If the method is applied to diagnosis, however, there are certain facts which should be kept carefully in mind. Such a method cannot be considered as a satisfactory substitute for the standard analytical procedures upon venous blood, for it is not possible to obtain the same degree of accuracy in measuring very small amounts as in measuring larger ones. The nature of the difference between capillary and venous blood must also be kept in mind when the results are interpreted. In the fasting condition it appears that there is no significant difference between the blood sugar concentrations in the two parts of the circulatory system,<sup>6</sup> but after the ingestion of carbohydrate this difference is often large<sup>6-9</sup> and an interpretation of results obtained upon such material in terms of normal values based upon analyses of fasting venous blood might easily lead to mistakes in diagnosis. Conservative interpretations must certainly be made after food has been ingested.

A method for the determination of sugar in very small samples of blood (0.02 c.c.) is described. The method is based upon a modification of the technique of Folin and Malmros. The results obtained agree closely with those of duplicate analyses by the method of Folin and Wu.

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## A COMPARISON OF METHODS USED IN THE DETECTION OF THE SICKLE-CELL TRAIT\*

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**M**ETHODS have been advocated for the detection of the sickle-cell trait. These include the following:

1. *Moist Preparation* (Emmel, Quoted by Cook and Meyer,<sup>1</sup> and Emmel<sup>2</sup>). A drop of capillary blood is sealed under a cover slip and examined microscopically at intervals thereafter. A change of shape of the erythrocytes from discoid to bizarre "fish-fin" and multipointed forms is diagnostic of sickleemia or the sickle-cell trait. The moist preparation has been widely adopted and is the method most widely used in diagnosis and in surveys of the sickle-cell trait.

2. *Gas Chamber Method* (Hahn and Gillespie<sup>3</sup>). A suspension of blood in citrated saline is placed on a cover slip and inverted over a small gas chamber. The edges are sealed. Carbon dioxide is passed through the chamber, and the shape of the cell is observed microscopically.

3. *Moist Stasis Preparation* (Sriver and Waugh<sup>4</sup>). A rubber band is placed around the proximal portion of the finger and allowed to remain for five minutes. The distal end of the finger is then punctured, and a drop of the dark blood is sealed under a cover slip (Fig. 1). Sriver and Waugh noted that the red blood cells sickled more rapidly when collected under such anoxic conditions than when the blood was collected without stasis.

4. *Test Tube Method* (Beck and Hertz<sup>5</sup>). A drop of finger blood is allowed to fall in a saline citrate solution in a test tube. Paraffin oil is added to prevent exposure to air. The preparation is allowed to stand for twenty-four hours at

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room temperature. Formaldehyde, 10 per cent in normal saline, is then added to fix the cells and to prevent their reversion to the round form on exposure to air. Moist preparations of the fixed cell suspension are examined microscopically.

5. *Moist Preparations Plus Dyes and Other Chemicals* (Hansen-Pruss<sup>6</sup>). Clean slides are covered with a thin film of chemical in solution, and the film is allowed to dry. A drop of blood from the finger is taken on a clean cover slip and placed on the prepared slide. The preparations are sealed with vaseline and examined microscopically.

Hansen-Pruss noted that the erythrocytes sickled faster and that the number of positive cases detected in an unselected group of negro patients was greater when brilliant cresyl blue, Janus green, methylene blue, and sodium cyanide were used than when the blood was untreated or when neutral red was used.

Because of the various methods advocated and the lack of evidence supporting the superiority of one method over another, a comparative study of the various methods used for the detection of the sickle-cell trait was made.

#### GAS CHAMBER METHOD

The gas chamber method is an ingenious method for the study of the factors involved in the sickling of erythrocytes, but it is impractical as a routine procedure. Special equipment and skill in manipulation are required. In our hands, the gas chamber method was less reliable than the ordinary moist, stasis moist, and test tube methods. Graham and McCarty,<sup>7</sup> and Scriver and Cooley,<sup>8</sup> and others have likewise found the gas chamber method to be unsatisfactory.

#### MOIST NONSTASIS AND MOIST STASIS WITHIN ONE HOUR

Two moist stasis and two moist nonstasis preparations were made on 406 unselected colored patients at the John Gaston Hospital. The preparations were examined within one hour after being made. One hundred erythrocytes were counted in each preparation, and the percentage of sickled cells was noted. The moist stasis preparations revealed sickling within the first hour in 26 instances, whereas the moist nonstasis preparations revealed sickling in 10 instances. The percentage of sickled cells in the two preparations of blood taken from the same patients and made by the same technique varied. Great variations were noted in the number of sickled cells in preparations from different patients. The number of sickled erythrocytes was significantly greater in the moist stasis preparations than in the moist nonstasis preparations. The average percentage in the 26 positive stasis preparations was 35 per cent, whereas the average percentage of sickled cells in the 10 positive nonstasis preparations was less than 1. In no instance did the nonstasis preparation show sickling in excess of the stasis. From these observations it is concluded that the stasis moist preparation is superior to the moist nonstasis preparation when the readings are made within one hour.

## MOIST NONSTASIS, MOIST STASIS, AND TEST TUBE METHODS IN TWENTY-FOUR HOURS

On each of the 406 unselected colored patients noted, a test tube preparation was made along with the two moist nonstasis and two moist stasis preparations. The technique as advocated by Beck and Hertz,<sup>5</sup> was followed as carefully as possible.

Of the 406 unselected cases, 37 were found to have erythrocytes possessing the ability to sickle in at least one of the preparations, an instance of 9.1 per cent. Of the 37 positive cases, the moist stasis was positive in 36 instances. With the test tube method the readings were positive in 35 instances. With the moist nonstasis preparations there were 31 instances of sickling (Table I).

The percentage of cells sickled in twenty-four hours in the stasis preparations was greater than in the test tube in 23 instances, less than in the test tube in 7 instances, and essentially the same in 6 instances. The average percentage of cells sickled with each method at the end of twenty-four hours is given in Table I.

TABLE I

METHODS	ONE HOUR		24 HOURS	
	INCIDENCE	AVERAGE % SICKLING	INCIDENCE	AVERAGE % SICKLING
Stasis	26	35	36	71
Nonstasis	10	1	31	45
Test tube			35	46

In two instances in which the stasis moist preparation was positive and the test tube was negative, the percentage of sickled cells in the stasis moist preparations was 94 and 20. In one instance in which the stasis preparation was negative and the test tube was positive, the percentage of cells sickled in the test tube was less than 1 per cent.

From these observations it is concluded that the stasis moist preparation and the test tube methods are more reliable than the moist nonstasis method for the detection of the sickle-cell trait, and that the stasis moist preparation and the test tube methods are of about comparable reliability. On account of the simplicity of the stasis moist preparation in comparison to the test tube method and the fact that the reading in the stasis moist preparation can often be made immediately, the stasis moist preparation is considered to be superior to the test tube method as a routine procedure.

## VITAL DYE, MOIST STASIS, MOIST NONSTASIS, AND TEST TUBE METHODS

An attempt was made to repeat the work of Hansen-Pruss<sup>6</sup> and to compare the stasis, nonstasis, and test tube methods with the vital dye moist preparation method. A comparison was also made between the stasis vital dye moist preparation and the nonstasis vital dye moist preparation (Table II). The vital dyes were prepared and placed on the slides as outlined by Hansen-Pruss, except that 95 per cent alcohol instead of absolute alcohol was used. The moist preparations were examined microscopically immediately after returning from the ward to the laboratory, and a second reading made the next day. The test tube preparation was made with blood from the nonstasis finger, and the reading was made at the end of twenty-four hours.

In this series of experiments the blood of 51 unselected colored patients was examined; sickling was demonstrated in 8. The percentage of sickled cells in each preparation made from the 8 patients are given in Table II.

TABLE II

CASES	MOIST STASIS					MOIST NONSTASIS					TEST TUBE
	NO DYE	METH. BLUE	NEUT. RED	B. C. BLUE	JANUS GREEN	NO DYE	METH. BLUE	NEUT. RED	B. C. BLUE	JANUS GREEN	
<i>Percentage of Erythrocytes Sickled Within 1 Hour</i>											
1	90	20	25	0	20	0	0	0	0	0	
2	30	90	40	0	90	4	85	0	0	60	
3	91	90	91	0	75	4	30	3	0	6	
4	100	100	93	0	100	93	100	91	0	95	
5	10	1	1	0	0	0	1	0	0	0	
6	10	1	1	0	0	0	1	0	0	0	
7	70	65	60	0	60	10	6	3	0	6	
8	80	85	75	0	80	20	25	10	0	10	
Average	69	69	58	0	53	22	41	14	0	22	
<i>Percentage of Erythrocytes Sickled Within 24 Hours</i>											
1	92	96	94	94	90	54	96	0	0	35	85
2	93	98	50	15	97	30	93	H	1	93	94
3	92	H	93	0	H	74	85	H	0	0	0
4	100	100	95	0	100	95	100	94	0	100	79
5	85	81	82	0	H	84	89	56	0	H	80
6	80	88	93	0	70	75	80	70	0	70	75
7	80	80	80	0	70	75	80	70	0	70	75
8	100	100	95	0	90	100	100	93	0	90	70
Average	90	91	85	14	89	72	91	67	0.12	78	68

H = Hemolyzed.

A study of the findings in this table reveals that brilliant cresyl blue inhibits the sickling of erythrocytes. This is in agreement with our past experience with brilliant cresyl blue, for we have noted in hundreds of brilliant cresyl blue moist preparations of blood from patients with sickle-cell anemia in which the reticulocytes were being studied that the sickling was not as marked as it was in the moist preparations in which no dye was used.

The sickling was more marked in methylene blue moist preparations than in Janus green or neutral red moist preparations.

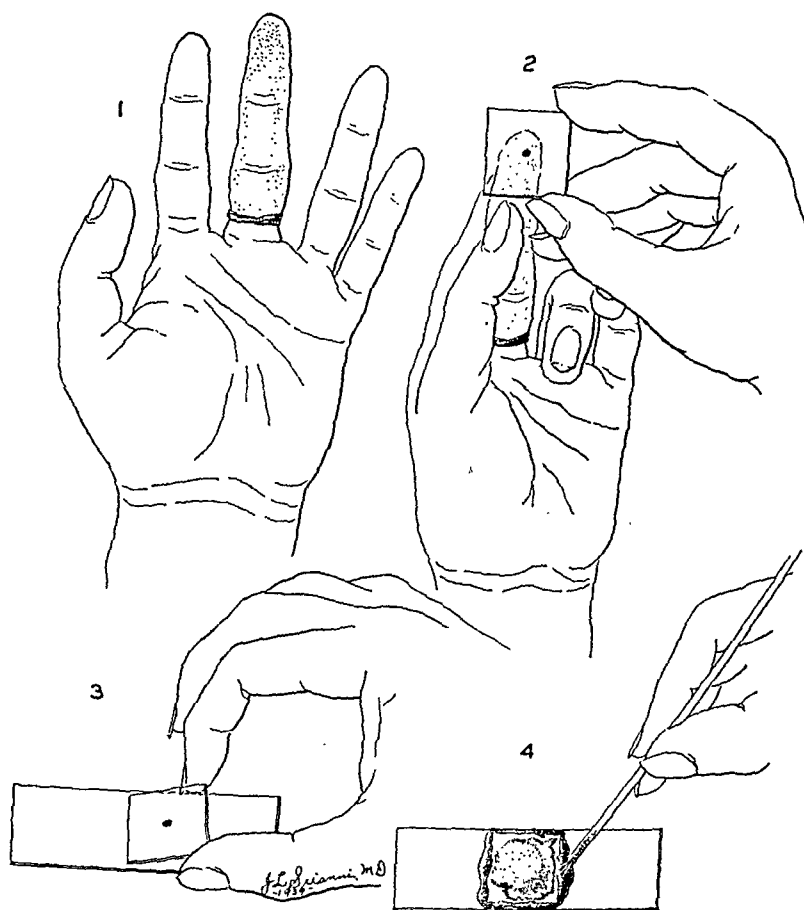
The stasis in the absence of dyes was more effective in producing sickling than dyes without stasis. The percentage of cells sickled in preparations with both dyes and stasis was no greater than in preparations with stasis in which no dyes were used.

The stasis method in this series yielded a greater percentage of sickled cells than did the moist nonstasis or the test tube methods.

#### COMMENT

The test tube method of Beck and Hertz yields sickled erythrocytes which are preserved in formalin and which are clearly defined and readily diagnosed. For the preservation of cells for teaching purposes, and for experimental studies of the effect of various substances on the sickle-cell phenomenon, the test tube method is recommended. As a routine method of diagnosis of sicklemia, however, the test tube method is inferior to the stasis moist preparation, for it is a little less reliable, and it is much more complicated, expensive, and time-consuming.

The use of dried films of dyes and other chemicals on slides as a simple means of studying the sickle-cell phenomenon and the factors that influence the sickling of erythrocytes is an excellent one. The use of methylene blue, Janus green, or neutral red as a routine procedure, however, has no apparent advantage over the stasis moist preparation; it has the disadvantage of being a more expensive and less practical method.



Figs. 1-4.—Stasis moist preparation for the detection of the sickle-cell trait.

The ordinary moist preparation is the simplest of all, but it is less reliable and requires a longer period of waiting before the reading can be made than does the moist stasis.

It is recommended that the moist stasis preparation be read whenever convenient after the preparation is made, but that the preparations in which the reading is negative within the first few hours be left for twenty-four hours at room temperature before the final reading is made. It is also advisable to make more than one moist stasis preparation, for sometimes the blood does not spread well, the seal is not complete, or the cells become hemolyzed. One should also transfer the blood from the puncture wound to the slide rapidly and prevent in so far as possible the exposure of the drop of blood to the air.

## CONCLUSION

The most reliable and the most practical method for the detection of the sickle-cell trait which has yet been devised is the moist stasis method of Scriven and Waugh.

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THE USE OF A SINGLE ANIMAL FOR TESTING THE  
VIRULENCE OF *C. DIPHTHERIAE*\*

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IN 1924 Fraser and Wigham<sup>1</sup> published a preliminary note on the use of intracutaneous tests on rabbits for determining the potency of diphtheria toxin and the virulence of diphtheria cultures. They reported that the variation between the reactions on individual rabbits was slight, and advocated the use of a dose of antitoxin five hours after the tests were placed, in order to preserve the animal at least until the completed reaction could be observed. A control test, using a known virulent strain, was included. They further recommended the use of the rabbit as assuring economy in time and animals, facility in reading reactions, and uniform basis for comparing several reactions on the same rabbit.

In 1926 Fraser and Weld<sup>2</sup> described a technique for intracutaneous testing on rabbits whereby a single animal could be used both as a test and as a control animal. This was accomplished by the intravenous administration of a large protective dose of antitoxin five hours after the test injections were placed, after which the test culture suspensions were injected into parallel skin areas on the other side of the rabbit. The rabbit was converted into a control by this dose of antitoxin, and the test was read by comparing the reactions on the two sides.

The use of the technique described by Fraser and Weld (1926) for determining the relationship between toxin production and intracutaneous virulence of recently isolated diphtheria cultures was reported in 1931 by

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Mader and Halpern.<sup>3</sup> These workers reported a variation of from 10 to 40 mm. in the diameter of the lesion in the cultures tested. They also used a known virulent strain as a control. This control culture, according to Mader and Halpern, yielded a "fairly constant reading between twenty-five and thirty millimeters in redness, hemorrhage and necrosis."

Fraser, Halpern, and Roy<sup>4</sup> in 1939 introduced another modification of the technique for the testing of virulence of diphtheria cultures by the intracutaneous inoculation of a rabbit. A mixture of culture suspension and antitoxin was inoculated into another skin area on the rabbit at the same time as the test culture suspension, an obvious saving in time and animals. A survey of diphtheria strains isolated from cases in California was in progress at the time this new modification of technique was described. Because many strains were to be tested for virulence and because white rabbits were available, the 1939 modification of the technique was adopted in place of the intracutaneous test on guinea pigs used in the department of hygiene since 1922<sup>5, 6</sup> for tests on both pure and field (mixed) cultures of diphtheria.

Pure cultures, isolated as single colonies from potassium tellurite-blood agar plates, were cultivated on Loeffler's medium. The growth, after forty-eight hours' incubation, was suspended in Stone's solution.<sup>7</sup> The use of this solution insures the retention of the toxin in the skin as it is formed. The culture suspension was divided and placed in two test tubes; to one portion diphtheria antitoxin was added (0.2 c.c. containing approximately 400 units for each 2.5 c.c. of test suspension), so that the mixture served as a control suspension; the unneutralized portion was the test suspension. One-tenth cubic centimeter of each portion was injected into rabbits by the intracutaneous route. The control was placed approximately 2 cm. below the test suspension. Female white rabbits were used so that the hair could be plucked from the sides, thereby insuring a relatively unirritated skin area for inoculation. This method for preparing the skin is superior to clipping, shaving, or the use of a depilatory for producing an area adequate in size and condition with the least effort on the part of the worker or discomfort to the animal. The tests were observed at twenty-four-hour intervals for four days. The occurrence of a red, indurated, and necrotic area at the site of injection of the test suspension, and of redness and induration only at the site of the injection of the test suspension, was interpreted as a positive reaction caused by the toxin produced by virulent diphtheria bacilli. The reaction was considered inconclusive when more induration, without necrosis, was observed in the test than in the control injection. A negative reaction was recorded when areas of redness and induration, which decreased in intensity after twenty-four hours, were noted at both injection sites.\*

Since the morphologic characteristics and cultural reactions of all the cultures tested had been typical of true *C. diphtheriae*, the occurrence of negative and inconclusive virulence tests by this new technique indicated the need for retesting with the technique familiar to the workers in this laboratory. All cultures which had yielded inconclusive reactions produced positive re-

\*The intracutaneous injection of Stone's solution alone will induce a red and indurated area which fades rapidly.



actions both on guinea pigs and on other rabbits when retested. Cultures that had yielded unquestionably negative reactions on rabbits were then included in the group, subject to retesting on other rabbits and guinea pigs. Upon retesting, some of these were positive on both test animals. Subsequently, several sets of tests, with freshly isolated strains, were done in duplicate on rabbits and singly on guinea pigs. It was observed that certain rabbits exhibited negative and inconclusive reactions to cultures which induced positive reactions on other rabbits and on guinea pigs. In order to discover what proportion of variation occurred in tests on rabbits a series of experiments were undertaken.

*Test Cultures.*—A set of 16 pure cultures of virulent diphtheria bacilli was selected; thirteen of these cultures had varied in reaction on two or more rabbits but had produced positive virulence tests on guinea pigs. Three cultures of the *gravis* type, which had always reacted positively on all rabbits and guinea pigs, were included as controls. Four *gravis*, five *intermedius*, and seven *mitis* strains were used.

*Preparation of Culture Suspension*—In order to obtain a sufficient amount of suspension, several slopes of Loeffler's medium were inoculated with each test culture. This was accomplished by delivering, with a pipette, portions of a saline suspension of the growth on a Loeffler's slope onto the surface of each of several slopes. Complete seeding of the surface was accomplished by rotating the inoculum carefully before placing the tubes in the incubator. After forty-eight hours' incubation the growth on each slope was suspended in 2 c.c. of Stone's solution. A pool of the suspensions of each test culture was divided into two parts, to one of which diphtheria antitoxin was added as in the preliminary tests. A stained smear from each test culture was examined to confirm the presence of diphtheria bacilli in pure culture. Subcultures were made from one tube of each test culture in order to verify the viability of the inoculum. Because of the color and consistency of the suspending solution, only a rough estimation of the relative opacity of the suspensions could be made.

*Test Animals.*—Female New Zealand white, New Zealand red, and Himalaya rabbits from several sources were used. All were mature animals, weighing 2,500 Gm. or more. Considerable difference in age was evident; variation in skin texture was noted. White guinea pigs, weighing 300 Gm. or more, were used. Most of these were females. The skin areas on the rabbits were prepared by plucking as previously described. The guinea pigs were plucked and shaved.

#### EXPERIMENTS

Four groups of animals were tested at ten-day intervals. Each set consisted of 16 to 18 rabbits and 8 to 16 guinea pigs. In each experiment some animals received test suspension only, so that the reaction to cultures could be observed in the absence of antitoxin in the skin. Those injected with both test and control suspensions were designated as test animals. Some of these test animals were given the control suspensions on the same side and below the test suspension, while others were given the test inoculations on the right side and the control inoculations on the left side. Sixteen tests were placed on the rabbits, and 8 on the guinea pigs. All injections were

made by one person who had had sufficient previous experience to assure the uniformity of the inoculations. The injection site was changed in each set of tests to eliminate the chance of one culture being repeatedly placed in an unfavorable skin area. The reactions were observed daily for four days.

In the first series 3 of 5 control rabbits died before twenty hours had elapsed. Thereafter all control rabbits received a subcutaneous following dose of 1,000 units of antitoxin five hours after the test injections were placed. Despite the use of a following dose of antitoxin, all control guinea pigs of the first series died in less than twenty hours. A new lot of antitoxin was used in the next two groups, but in twenty-four hours the control guinea pigs were either dead or so ill that the tests could not be accurately read. In the last experimental group the control guinea pigs were given 1,000 units of antitoxin as a following dose. All these guinea pigs lived at least forty-eight hours.

Reactions were classified as positive when the central area of necrosis, which finally became scabbed, was 3 mm. or more in diameter. Reactions were classified as inconclusive when the necrotic area was less than 3 mm. in diameter, but the development of the lesion was at the same rate as positive tests on the same animal. Reactions were classified as negative when no necrosis was observed, or when no reaction could be detected after three days.

TABLE I

REACTION OF ANIMALS TO THE INTRACUTANEOUS INOCULATION OF A TEST SERIES OF CULTURES OF *C. DIPHTHERIAE*

	CONTROL ANIMALS		TEST ANIMALS		TOTAL
	ALL REACTIONS POSITIVE	VARIABLE REACTIONS	ALL REACTIONS POSITIVE	VARIABLE REACTIONS	
Rabbits	13	3	7	29	52
Guinea pigs	16	1	14	9	40

## RESULTS

Fifty-two rabbits received intracutaneous injections of a set of pure cultures of diphtheria bacilli (Table I). Sixteen of these, serving as controls, received injections of test suspensions alone. All tests were positive on 13 of these rabbits, while 3 exhibited inconclusive or negative tests with culture suspensions that yielded positive reactions on other rabbits and on guinea pigs. Thirty-six rabbits were used as test animals, receiving both test and control suspension. Twenty-nine of the 36 rabbits showed inconclusive or negative reactions to the injection of test suspensions which induced positive reactions on control rabbits and guinea pigs, whereas only 7 reacted positively to all injections.

Tests on guinea pigs yielded more uniform reactions (Table I). Seventeen control guinea pigs were inoculated with test suspensions. On 16 all reactions were positive. Only one guinea pig showed variation in reaction to the inoculation of virulent diphtheria cultures. Twenty-three guinea pigs received injections of both test and control suspensions. Nine of these animals showed one

or more negative or inconclusive reactions to test suspensions which induced positive reactions in control rabbits and guinea pigs, while 14 reacted positively to all tests.

Two test strains (both intermediate type) frequently gave inconclusive or very small reactions on control animals. When test animals were injected with these strains, the reactions were usually inconclusive or negative, although large typical reactions were observed on a few test and some control animals. Two of the 3 gravis control strains were uniformly positive reactors on all animals tested. Each of the other 12 test strains reacted positively on some animals in an experimental group, but gave negative or inconclusive reactions on other animals in the same or another group.

Differences in size, age, breed of rabbit, skin area injected, or texture of the skin could not be correlated with the variation in reactivity of the individual rabbit to virulent diphtheria bacilli. Of the 29 test rabbits (injected with both test and control suspensions) from 2 to 14 tests were inconclusive or negative on a single rabbit. Marked differences in the rate of development of lesions on individual animals were noted. The differences in size and character of the lesions in their early stages were especially noticeable. The reactions on the control rabbits were uniformly and obviously larger than those on the test rabbits, though the same variation in rate of development and character of lesion was evident in individual rabbits in both test and control groups.

The reactions on control guinea pigs were larger than those on test guinea pigs. Variation in the rate of development in individual guinea pigs was noted, but there was less difference in the character of the lesion in guinea pigs than in rabbits. No difference in reaction was observed in rabbits or guinea pigs when the control suspensions were inoculated on the same side as the test suspensions or when all control suspensions were inoculated on one side.

#### DISCUSSION

Previously intracutaneous tests for potency of vaccinia virus (unpublished data) had shown such variation in rabbit reactions that these results with *C. diphtheriae* were not unexpected. It is evident that individual rabbits may fail to react to the injection of virulent diphtheria bacilli so that virulence tests on such animals might be falsely reported as negative. The use of a control test culture will not solve the problem since, in these experiments, only 2 of 16 virulent cultures reacted positively on all rabbits.

The use of one rabbit for a set of tests, which is made possible by the injection of a control suspension, evidently increases the hazard inherent in the use of rabbits as test animals. Only 7 of 36 rabbits yielded positive results in all tests with pure cultures of diphtheria bacilli. It is reasonable to suppose that the amount of antitoxin in the control suspensions is responsible for the fact that more than twice as many test as control rabbits failed to react to the intracutaneous injection of *C. diphtheriae*. The technique described by Fraser and his co-workers decreases the reliability of the intracutaneous virulence test on rabbits.

The guinea pigs inoculated in these experiments were primarily intended as a control group. As variation in the reactions on test guinea pigs was noted, the number of animals was increased beyond the original plan. The results of these experiments are suggestive, but many more tests must be made in order to discover how frequently individual guinea pigs vary in their response to the inoculation of suspensions of the diphtheria bacillus. The public health laboratory technician is frequently called upon to test a few cultures for virulence. It would be economy in time and animals if one guinea pig could be used for these tests, as would be possible if both test and control suspension were inoculated at the same time. Since it seems evident that guinea pigs exhibit less variation, it may be possible to discover a safe adaptation of the method by reducing the amount of antitoxin in the control inoculum and the number of tests performed on one animal. This problem is being studied at this time and will be the subject of a later report.

#### CONCLUSIONS

1. The rabbit is not a suitable animal for the assay of virulence of *C. diphtheriae* by intracutaneous inoculation.

2. The use of a single rabbit for virulence testing by the simultaneous inoculation of test culture suspensions and control suspensions containing antitoxin does not yield reliable results.

3. Experimental evidence is presented which suggests that guinea pigs are less subject to variation in reaction than are rabbits. This will be investigated further.

4. The simultaneous intracutaneous inoculation of test culture suspensions and control suspensions containing antitoxin causes considerable variation in the reaction of guinea pigs but less than that noted in rabbits. This evidence corroborates that presented by the experiments with control guinea pigs which indicates that individual guinea pig variation may occur.

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## RAPID METHOD FOR THE ISOLATION OF KERASIN FROM A GAUCHER SPLEEN\*

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**K**ERASIN is a characteristic component of the lipid fraction in Gaucher's disease and is used as a chemical confirmation of the diagnosis. For this reason, it is desirable to develop simple methods for its isolation and identification.

Kerasin is usually isolated from organs after preliminary drying and extraction of lipids. Rosenheim<sup>1</sup> accomplished both processes in one step by repeated extractions of wet organs with acetone. The kerasin was then isolated by extracting the dried residue with warm pyridine. More recently, McConnell, Forbes, and Apperly<sup>2</sup> extracted a dried spleen from a case of Gaucher's disease with acetone and ether repeatedly until the residue was lipid-free, and then extracted the kerasin from the residue with hot alcohol.

I employed both methods for the extraction of kerasin from a Gaucher spleen. The latter method gave a purer product. At the same time a method was devised for the extraction of kerasin which would entail no previous drying or repeated extractions with acetone and ether. It was found that kerasin could be removed completely and in a very pure state simply by drying the spleen with plaster of Paris and extracting the dried organ with hot 95 per cent alcohol. The alcohol on cooling deposited a bulky white precipitate of kerasin.

### PROCEDURE

Plaster of Paris was added slowly and mixed with about 500 Gm. of finely ground spleen until a thick paste was formed. This was allowed to set for about an hour, then ground to a fine powder in a mortar. The powder was then extracted with 1,500 ml. of boiling 95 per cent ethyl alcohol. The suspension was filtered while hot through a large Buchner funnel, and the filtrate was cooled to 0° C. in a refrigerator. The precipitate formed on cooling was filtered with the aid of suction, and the alcoholic filtrate was used for further extractions of the splenic material. This process was repeated seven more times until no precipitate was formed on chilling the alcohol.

The precipitates of almost pure kerasin were combined and freed of lipid impurities by extraction over a period of four hours with ether in a Soxhlet extraction. The melting point of the dried material was found to be about 201° C. Repeated recrystallizations from methyl and ethyl alcohols gave a material which started to melt at 189° C., and melted with decomposi-

\*From the Pediatric Research Laboratory, the Jewish Hospital of Brooklyn, Brooklyn.  
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tion at 204° C. Further purification, following the method of Bloom and Kern,<sup>3</sup> gave a product with the same melting point. A positive selenite test was obtained according to the procedure of Rosenheim.<sup>1</sup>

Hydrolysis with sulfuric acid in methyl alcohol gave a precipitate which, upon recrystallization from acetone, melted at 57° C. This corresponds closely with the melting point of the methyl ester of lignoceric acid which Rosenheim prepared by the same procedure and which was found to melt at 57° to 58° C.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**RENAL DISEASE, Urea Reabsorption and Relation Between Creatinine and Urea Clearance in, Arkin, A., and Popper, H.** *Arch. Int. Med.* 65: 627, 1940.

The clearance of urea and that of endogenous creatinine were compared in 87 cases of normal and of pathologic conditions in order to estimate the reabsorption of urea.

Urea reabsorption is constant during the same day, but not on different days. It is slightly diminished during fasting in moderate glomerular disorders, such as acute glomerulonephritis, benign hypertension, nephrosis, and extrarenal glomerular insufficiency. It is markedly diminished in many cases of severe damage to the kidneys, as in chronic nephritis, malignant hypertension, pyelonephritic contracted kidney, and extraglomerular acute nephritis (1 case). The lowest rate of reabsorption was found in pyelonephritic contracted kidney.

The decrease in urea reabsorption, usually combined with disturbed filtration and reabsorption of water, may depend on two factors: the decrease in the urinary concentration and the presence of damage to the kidneys.

With severest damage to the kidneys the urea clearance may exceed the creatinine clearance. This may be interpreted as an indication of urea secretion.

Uremia due to reabsorption in renal disease does not exist. On the contrary, stronger antiosmotic forces are effective at the tubular barrier against the back flow of urea. This mechanism is probably compensatory in order to prevent urea retention with decreased filtration.

**ANGIITIS, Experimental Infectious, Winternitz, M. C., and LeCompte, P. M.** *Am. J. Path.* 16: 1, 1940.

Various bacteria were introduced under aseptic technique into the adventitial tissue (more rarely on a thread into the lumen or into a doubly ligated stretch) of femoral, jugular, and carotid vessels of goats. By varying the virulence of the organism and the duration of the experiment, lesions of both artery and vein were obtained, ranging from acute suppurative and proliferative reactions with thrombosis to old, fibrous intimal plaques. A potential vascular pathway for passage of infection from vein to artery has been demonstrated in both goat and man by injection methods.

**RHEUMATIC FEVER, Weltmann Reaction and Sedimentation Time During, of Childhood, Klein, R. I., Levinson, S. A., and Rosenblum, P.** *Am. J. Dis. Child.* 59: 48, 1940.

The Weltmann reaction and the sedimentation rate were studied in 110 cases of rheumatic fever of childhood and in 10 cases of subacute bacterial endocarditis. The rheumatic fever group was made up of 26 cases of chorea, 32 cases of acute rheumatic arthritis without carditis, 39 cases of acute carditis, and 13 cases of cardiac decompensation.

1. The sedimentation rate with chorea, although usually normal, may at times be increased. This increased rate may be related to a previous infection. The Weltmann reaction with chorea is almost always either normal or increased.

2. Acute rheumatic arthritis and carditis are characterized by a rapid sedimentation rate and a low coagulation band. The coagulation band returns to normal before the sedimentation rate.

3. In cases of cardiac decompensation the sedimentation rate tends to slow, and the coagulation band tends to increase the values, depending on the severity of the decompensation in relation to the degree of infection.

4. The three pathologic stages of rheumatic fever, exudative, proliferative, and fibrotic, are reflected in the Weltmann reaction.

5. An increased sedimentation rate does not always mean that the rheumatic infection is still active. The patient may be convalescing in a phase which is pathologically termed the proliferative stage and still have a rapid sedimentation rate.

6. The Weltmann reaction is of value in the study of rheumatic fever, complementing the sedimentation rate; it may be of aid in the differentiation of subacute bacterial endocarditis from acute rheumatic carditis.

**LEAD POISONING, Glycosuria in, Goettsch, E., and Mason, H. H.** *Am. J. Dis. Child.* 59: 119, 1940.

A single case of lead poisoning with glycosuria is reported in detail. Studies carried out on this patient show that the height of the blood sugar was consistently normal or lower than normal, and that the urinary concentration of sugar remained relatively constant irrespective of the diet. The glycosuria persisted for five weeks, and the concentration of urinary sugar fell as convalescence progressed. The studies are compatible with the suggestion that the condition in lead poisoning may be classified as renal glycosuria. The suggestion is supported by a survey of 8 cases of lead poisoning with glycosuria in children. In 5 of the cases in which determinations were made, the blood sugar values were normal; in 3 cases in which dextrose tolerance curves were obtained, the curves were not of the diabetic type. Glycosuria does not seem to alter the prognosis in cases of lead poisoning in children.

**COMPLEMENT FIXATION, A Simplified Technic, Its Sensitivity and Specificity, Gillman, R. L., Boerner, F., and Lukens, M.** *Arch. Dermat. & Syph.* 41: 32, 1940.

The authors have described a new modification of the complement fixation test for syphilis, which has been determined to be more sensitive than the Kolmer modification and the Kahn flocculation test, although less so than the Eagle flocculation test. There has been no loss in specificity.

The Boerner-Lukens modification of the complement fixation test tends toward simplification through the use of an easily prepared antigen and the addition of the reagents in combination. There ensues less labor and cost and the addition of improved supervision.

In a series of 1,500 tests, there were 20 instances of lack of accord in specificity, all of which are analyzed against the clinical status of the patient.

The continuance of the use of two or three tests for the serologic evaluation of syphilis is urgent.

A closer rapport is necessary between the serologist and the clinician, in order that the patient's interests may best be served.

**SULFAPYRIDINE, Pathologic and Histologic Changes Following Oral Administration of, Antopol, W., and Robinson, H.** *Arch. Path.* 29: 67, 1940.

Urolithiasis occurs after the feeding of sulfapyridine to monkeys, rabbits, and rats. The concretions can be either redissolved or washed out. It cannot be ascertained whether the formation of the urolith is always an independent precipitation process, or whether it is at times dependent on primary degenerative or vascular changes in the kidney.

**TUBERCULIN TEST, The Patch Test and Mantoux Intradermal Test, Pearce, A. J., Fried, R. L., and Glover, V. A.** *J. A. M. A.* 114: 227, 1940.

Seven hundred and twelve school children were given both the tuberculin patch test and the Mantoux test, first and second strength purified protein derivative.

Six hundred and sixteen had either both tests positive or both negative, the percentage correlation between the two tests in this series being 87 plus.



Sixty-seven had positive patch and negative Mantoux tests.

The tuberculin patch test has a high degree of correlation with the Mantoux test and appears to give 7 per cent more positives than the Mantoux.

The authors conclude that the tuberculin patch test is as reliable as the Mantoux. Its ease of application and nontraumatizing character make it superior to the Mantoux test in other ways.

They believed for these reasons that the tuberculin patch test is the method of choice in large scale tuberculin testing, especially for children.

**CEREBROVASCULAR ACCIDENTS, Duration of Life After, Newbill, H. P. J. A. M. A 114: 236, 1940.**

Sudden death (within two hours) from cerebrovascular accidents is the exception rather than the rule.

Hemorrhage is more likely to be responsible if death occurs within twenty-four hours of the onset of symptoms, while thrombosis is far more common in patients surviving for more than one month.

The average survival period after thrombosis is approximately fifteen times as long as after hemorrhage or embolism.

There was a distinct difference in the survival periods of the white (105.5 days) and the Negro races (64.8 days). This difference was confined mainly to the patients with thrombosis.

There was an equally distinct difference between males (57.3 days) and females (129.1 days). Here again the essential difference was found to be in the group with thrombosis.

When apoplexy occurred between the ages of 21 and 60, the average survival period was one month or longer. Death usually occurred within one month when the patient was below 21 or above 60 years old. The maximal survival period fell within the 30 to 40 year group, here averaging 141.0 days.

As would be expected, the location of the lesion and the number of lesions were important determining factors in the survival period.

**PREGNANCY, Study of "False" Friedman Tests for, Randall, L. M., Magath, T. B., and Pansch, F. N. J. A. M. A. 114: 471, 1940.**

The Friedman test is based primarily on the fact that if anterior pituitary-like hormones are present in the urine in certain amounts, they will be demonstrable by typical reactions in the ovary of the rabbit. While the test is not a test of pregnancy from an academic point of view, to all practical purposes it is so if the correct technique is followed.

The Friedman test is, therefore, based on quantitative considerations. Excretion of the gonadotropic principle of the anterior lobe of the hypophysis in the urine in excess amounts may be responsible for a positive Friedman reaction in the absence of pregnancy.

Among the several physiologic and pathologic states other than normal pregnancy which may give a positive reaction are hydatidiform mole, chorio-epithelioma, the menopause, menstrual disorders, such as primary ovarian failure, treatment with preparations of the anterior lobe of the pituitary gland, and errors in technique.

A negative reaction to the Friedman test prior to the seventh week after the last menstrual period may not be conclusive, although authentic positive reactions may be obtained much earlier, frequently within four weeks after impregnation. A test that gives negative results before the seventh week should be repeated later.

**PNEUMOCOCCIC INFECTIONS, Treatment of, in Children With Sulfapyridine, Christian, H. S., Jorgensen, G. M., and Ellis, C. Am. J. Dis. Child. 59: 1, 1940.**

Sulfapyridine was used in the treatment of 140 patients with pneumococcic infections. No patients were left untreated for controls. Among the 100 patients with pneumonia only one death occurred. For 9 of the 13 patients with pneumococcic bacteremia the blood

## REVIEWS

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Books and Monographs for Review should be sent direct to the Editor,  
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

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### Diseases of the Skin\*

A BOOK which is still widely used twenty-three years after its first appearance, and which has gone through a total of ten editions, requires no introduction. The tendency of such volumes is to become progressively larger in succeeding editions. This book has followed this tendency, containing now over 1,500 pages. However, this does not imply simple additions to previous pages, for the present edition has been revised and reset. Its large size is merely evidence of the tremendous increase of knowledge and observations in the field.

The illustrations are startlingly good and most abundant. This is an essential feature of all good texts on dermatology.

New aspects in dermatology that have been adequately covered include the allergic dermatoses and dermatoses associated with constitutional maladies.

This is an excellent reference volume.

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### Mathews' Physiological Chemistry†

TWENTY-FIVE years ago "Mathews" was a standard text in physiological chemistry, used by the present reviewer in his student days. With the continuous avalanche of new discoveries and the more or less continuous changing of teachers, many of whom write and prescribe their own texts, a quarter century is a long life for any book in medicine. It must very nearly approach a record. The first edition appeared in 1915. From then until 1932 either a reprinting or a new edition appeared every year except 1929. Then came a lapse of several years, until the present sixth edition appeared in September, 1939.

One can follow in his imagination the mental attitude of an author who, struggling year after year in his successive revisions, at last becomes surfeited with what had started as a pastime and had ended as a task. After the lapse of sufficient time he again becomes interested and enthusiastic. The fresh approach enables him to start at the beginning, with no subconscious regrets at cutting widely in many places. The result is a textbook of biochemistry, up to date, which stands as a monument to a pioneer and leader in the field.

There are two general parts, with many subheadings. The first is "The Chemistry of Protoplasm and the Cell"; the second is "The Mammalian Body Considered as a Mechanism."

The section on Laboratory Outlines and Laboratory Methods has been omitted and is published separately by F. C. Koch. Even with this omission the volume contains nearly 1,500 pages.

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\*Diseases of the Skin. By Richard L. Sutton, M.D., Sc.D., LL.D., F.R.S. (Edin.), Professor of Dermatology, University of Kansas, School of Medicine; and Richard L. Sutton, Jr., A.M., M.D., L.R.C.P. (Edin.), Associate in Dermatology, University of Kansas, School of Medicine. Cloth, ed. 10, 1,549 pages, with 1,452 text illustrations and 21 color plates, revised, enlarged, and reset. The C. V. Mosby Company, St. Louis, Mo., 1939.

†Physiological Chemistry: A Text-book for Students. By Albert P. Mathews. Cloth, ed. 6, 1,488 pages, with 113 illustrations, \$8.00. William Wood & Co., Baltimore, Md., 1939.

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## CLINICAL AND EXPERIMENTAL

### EXPERIMENTAL STUDY OF THE EFFECTS OF SULFAPYRIDINE ON STAPHYLOCOCCI AND STAPHYLOCOCCUS TOXIN\*

R. H. RIGDON, M.D., AND P. R. FREEMAN, MEMPHIS, TENN.

THERE are only a few observations on either clinical or experimental use of sulfapyridine in staphylococcus infections.<sup>1-3</sup> Whitby<sup>2</sup> and Long<sup>3</sup> have found that this drug is an active chemotherapeutic agent in the treatment of staphylococcus infections in mice. The mode of action of sulfapyridine on staphylococci is not clearly understood.

This paper is a report of a series of experiments in which the in vitro action of sulfapyridine† was studied on three strains of staphylococci. It also includes a study of the action of sulfapyridine on staphylococcus toxin both in vitro and in vivo.

#### EFFECT OF SULFAPYRIDINE ON STAPHYLOCOCCI IN VITRO

Three strains of staphylococci were used in this experiment. Strain "A-O" is a variant from a culture of *Staphylococcus aureus*, isolated in 1933 from the pharynx of a patient with agranulocytic angina. It produces a yellow pigment, a toxin that lyses rabbit red blood cells, necrotizes rabbit skin, and is lethal for mice.

Strain "Wood" was obtained from Lederle Laboratories in 1937. It also produces a potent toxin that lyses red blood cells, necrotizes the skin of rabbits, and is lethal for mice. This strain of staphylococcus is hemolytic but does not produce pigment.

\*From the University of Tennessee, Department of Pathology, Memphis.  
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†The sulfapyridine was obtained through the courtesy of Lederle Laboratories.

Strain "7" was isolated from a furuncle in 1937. It is nonpigmented and does not produce any hemolysis when grown on blood agar plates containing rabbit red blood cells. This strain of staphylococci does not produce toxin.

These staphylococci were grown on nutrient broth medium (Difco) with 2 per cent dextrose. It was buffered, and the pH was adjusted to 7.2. Varying amounts of a 2 per cent solution of sulfapyridine in broth were added to a series of tubes containing this medium. The total volume of broth and sulfapyridine was 5.0 c.c. The tubes were autoclaved for twenty minutes at 20 pounds pressure. The following amount of sulfapyridine was put into each tube:

SERIES	QUANTITY OF SULFAPYRIDINE		Mg./c.c.
1	1.0	c.c. of 2.0 per cent solution	4.0
2	0.75	c.c. of 2.0 per cent solution	3.0
3	0.50	c.c. of 2.0 per cent solution	2.0
4	0.25	c.c. of 2.0 per cent solution	1.0
5	1.0	c.c. of 0.2 per cent solution	0.4
6	0.75	c.c. of 0.2 per cent solution	0.3
7	0.50	c.c. of 0.2 per cent solution	0.2
8	0.25	c.c. of 0.2 per cent solution	0.1
9	1.0	c.c. of 0.02 per cent solution	0.04
10	0.75	c.c. of 0.02 per cent solution	0.03
11	0.50	c.c. of 0.02 per cent solution	0.02
12	0.25	c.c. of 0.02 per cent solution	0.01

The first four series of tubes showed a small quantity of sulfapyridine in the bottom of each tube after autoclaving. The quantity of sulfapyridine precipitated decreased, however, with each dilution.

Four dilutions of each of the three strains of staphylococci were used to inoculate a group of the tubes containing the sulfapyridine as follows: (A) a twenty-four-hour broth culture; (B) a 1:10,000 dilution; (C) a 1:1,000,000 dilution; (D) a 1:10,000,000 dilution of the culture used in (A). A wire loop was used to transfer the inoculum. The medium was incubated at 37.5° C., and growth was observed at twenty-four-hour intervals. The quantity of growth in each tube is indicated by pluses, the greatest by ++++ and the least by +. Tubes are marked ? in which it was questionable whether or not there was any bacterial growth. No growth is indicated by 0.

The result of this experiment is shown in Chart 1. From these data it is evident that sulfapyridine has a bacteriostatic effect on each strain of staphylococci used in this study. The greatest effect was produced on strain "7." Blood agar plates were poured with a 1:10,000,000 dilution of each strain of staphylococci, and every plate showed too many colonies to count. Other experiments in which the colonies were counted have shown, however, that the number of bacteria per unit of inoculum will not account for this variation in growth of the three strains of staphylococci in the same concentrations of sulfapyridine.

The bacterial growth was smooth in all the tubes containing broth and sulfapyridine during the first forty-eight hours. At this time some of the tubes began to show a slightly granular appearance. Strain "Wood" showed this change more than either "A-O" or "7." The number of granules progressively increased and accumulated in the bottom of the tubes. An apparent diminution in growth accompanied the formation of granules in the medium.

This granular type of growth observed with the three strains of staphylococci suggests that some change may have occurred in the organisms during the

TIME	STRAIN OF STAPH	DILUTION	BACTERIAL GROWTH											
9 AM. 5/25	A-O	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	WOOD	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	#7	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
8 AM 5/26	A-O	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	WOOD	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	#7	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
11 AM 5/27	A-O	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	WOOD	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	#7	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2 P.M. 5/28	A-O	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	WOOD	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	#7	1:1000000	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
MG. SULFAPYRIDINE PER CC.			40	30	20	10	4	.3	.2	.1	.04	.03	.02	.01
A-24 HOUR BROTH CULTURE UNDILUTED														
B-A' DILUTED 1:100000														
C-A' " 1:1000000														
D-A' " 1:1000000														
O-NO BACTERIAL GROWTH														
?-QUESTIONABLE BACTERIAL GROWTH														
- NO OBSERVATION														
****MAXIMUM BACTERIAL GROWTH WITH PROGRESSIVE DIMINUTION OF GROWTH INDICATED BY ****														

Chart 1.—The effect of different concentrations of sulfapyridine on the growth of three strains of staphylococci in vitro.

period in which they were in the presence of sulfapyridine. To determine whether the organism had been changed by the action of sulfapyridine, three groups of 14 different sugars in broth were prepared. These sugars were inoculated with each of the three strains of staphylococci which had been growing in the presence of sulfapyridine for forty-eight hours. The control inoculum was the same three cultures grown in only extract broth for twenty-four hours. The fermentation reactions after forty-eight hours were the same for the group of staphylococci grown in the presence of sulfapyridine as they were for the staphylococci in the control medium.

TABLE I  
EFFECT OF SULFAPYRIDINE ON THE HEMOLYSIS IN STAPHYLOCOCCUS TOXIN

2.0 c.c. staphylococcus toxin + 100 mg. sulfapyridine	DEGREE OF LYSIS AFTER EIGHTEEN HOURS. QUANTITY OF STAPHYLOCOCCUS TOXIN, C.C.														
	0.08	0.07	0.05	0.03	0.02	0.01	0.008	0.007	0.005	0.003	0.002	0.001	0.0008	0.0007	0.0005
Sulfapyridine	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Control	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4

4 = Complete lysis of red blood cells.  
3 = 75 per cent lysis of red blood cells.  
2 = 50 per cent lysis of red blood cells.  
1 = 25 per cent lysis of red blood cells.

TABLE II  
EFFECT OF SULFAPYRIDINE ON RED BLOOD CELLS IN THE PRESENCE OF STAPHYLOCOCCUS TOXIN

Quantity of sulfapyridine with rabbit red cells for 2 hr.	DEGREE OF LYSIS AFTER TWENTY-FOUR HOURS. QUANTITY OF STAPHYLOCOCCUS TOXIN, C.C.														
	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001	0.0009	0.0008	0.0007	0.0006	0.0005	0.0004
0.1 mg. per c.c.	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2
1 mg. per c.c.	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2
5 mg. per c.c.	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2
Control	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2

The red blood cells were suspended in saline in 1 per cent concentration.

4 = Complete lysis of red blood cells.  
3 = 75 per cent lysis of red blood cells.  
2 = 50 per cent lysis of red blood cells.  
1 = 25 per cent lysis of red blood cells.

The failure of strain "7" to grow in a concentration of sulfapyridine in which "A-O" and "Wood" grew luxurantly, as shown in Chart 1, suggests that this chemical exerts a greater bactericidal action on one strain of staphylococci than upon another. The absence of any growth after seventy-two hours in certain tubes inoculated with strain "7" suggests that sulfapyridine may be bactericidal for certain staphylococci when the organisms are present in small numbers. Each tube inoculated with strain "7" in groups B and D, as shown in Chart 1, was cultured. The tubes were shaken, and a wire loop of inoculum was placed in extract broth. The only tubes showing a positive growth after forty-eight hours' incubation were the tubes inoculated from those showing a definite growth. These results suggest that the bacteria in these tubes apparently were dead at the time the second group of tubes was inoculated.

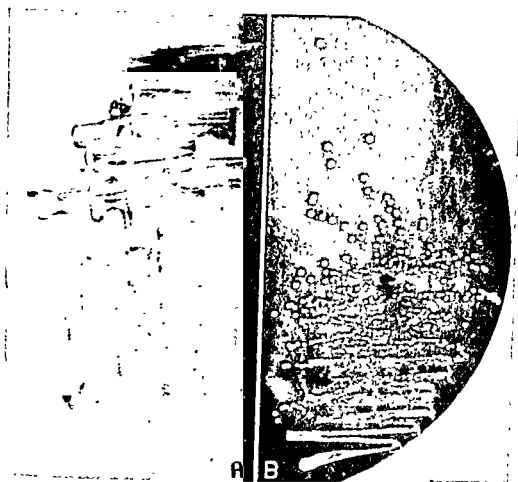


Fig. 1.—Colonies of staphylococci strain "7" on surface of blood agar plates. A, Plate contains 10 mg. of sulfapyridine per cubic centimeter of blood agar. B, Control for A. Photographed after twenty-four hours.

A second experiment was made to study the effect of sulfapyridine on the growth of staphylococci. In this, three different concentrations of sulfapyridine, 1, 5, and 10 mg. per c.c., were added to infusion blood agar. An equal quantity of this blood agar was then put into a group of Petri dishes. The control was blood agar with no sulfapyridine.

The control medium and the three groups of Petri dishes with sulfapyridine in the blood agar were inoculated with strains "A-O," "Wood," and "7." The plates were incubated at 37.5° C. for twenty-four hours. There was a marked diminution in the size of the colonies on the blood agar plates with 5 and 10 mg. per c.c. of sulfapyridine when compared with the controls. The

colonies from strain "7" were also much smaller than those inoculated with "A-O" and "Wood." Fig. 1 shows the colonies from strain "7" after growing on blood agar for twenty-four hours in the presence of 10 mg. per c.c. of sulfapyridine; *A* shows the bacteria growing in the presence of sulfapyridine; *B* is the control.

The amount of hemolysis that occurs around the individual colonies is decreased in those plates with sulfapyridine as compared with the controls. It is interesting to note, however, that the width of the hemolytic zone around the colonies is always proportional to the size of the colony.

#### EFFECT OF SULFAPYRIDINE ON HEMOLYSIS PRODUCED BY STAPHYLOCOCCUS TOXIN

In the preceding experiments it was found that hemolysis occurred around staphylococcus colonies growing in the presence of sulfapyridine. The present experiment is a further study of the effect of this drug on the hemolytic, the skin necrotizing, and the lethal factor in staphylococcus toxin.

One hundred milligrams of sulfapyridine were added to 2 c.c. of staphylococcus toxin. Only a small amount of the drug was soluble even after thorough shaking. The toxin-sulfapyridine mixture remained together for fifteen minutes and was then titrated. Washed rabbit red blood cells in a 1 per cent saline suspension were used in the hemolytic titrations. The results of this experiment are shown in Table I. From this table it can be seen that there is no difference in the degree of lysis produced by staphylococcus toxin alone and by staphylococcus toxin in the presence of sulfapyridine for fifteen minutes before titration.

To study further the action of sulfapyridine on the hemolysis produced by staphylococcus toxin, the following experiment was performed: Red blood cells from a rabbit were carefully washed four times in large volumes of saline. One cubic centimeter of these washed cells was put into each of four flasks containing 100 c.c. of saline. Sulfapyridine in quantities of 10 mg., 100 mg., and 500 mg., respectively, was added to three of the flasks; the fourth flask was the control. The red blood cells remained in the presence of the sulfapyridine for two hours, after which time 1 c.c. of the different suspensions of red blood cells was added to a series of tubes containing decreasing amounts of staphylococcus toxin. The results of this titration are shown in Table II. It is evident from these data that rabbit red blood cells treated with various concentrations of sulfapyridine for two hours react to the hemolytic action of staphylococcus toxin the same as red blood cells suspended in saline.

The failure of sulfapyridine to inhibit hemolysis is also shown by the following experiment: A group of blood agar plates, containing varying amounts of sulfapyridine, was prepared in the same manner as those previously described in this paper. Three drops of staphylococcus toxin were carefully dropped from a capillary pipette onto the surface of the plates. After two hours the toxin was absorbed, and the plates were placed in the incubator at 37.5° C. There was no difference in the size of the hemolytic zones produced by the toxin on the plates containing sulfapyridine and the control medium. This hemolysis is shown in Fig. 2. The blood agar in *A* contains 10 mg. per cubic centimeter of sulfapyridine, while *B* contains no sulfapyridine.



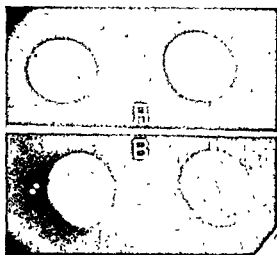


Fig. 2.—Hemolysis produced by staphylococcus toxin on blood agar plates. A, Plate contains 10 mg. of sulfapyridine per cubic centimeter of blood agar B, Control for A. The degree of hemolysis is the same on both plates after twenty-four hours.

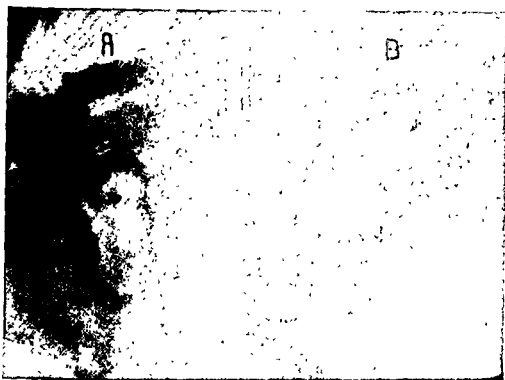


Fig. 3.—Skin necrosis produced by the intradermal injection of staphylococcus toxin in the rabbit. One-tenth cubic centimeter of toxin diluted 1:10 with saline was injected at A. A similar amount of toxin with 10 mg. sulfapyridine per cubic centimeter was injected at B. Photographed after twenty-four hours.

#### EFFECT OF SULFAPYRIDINE ON SKIN-NECROTIZING FACTOR IN STAPHYLOCOCCUS TOXIN

Four rabbits were used in this experiment. The skin was carefully shaven twenty-four hours or longer before the toxin was injected intradermally. Duplicate injections were made into each animal. The staphylococcus toxin was diluted in saline in which there was 10 mg. of sulfapyridine per cubic centimeter. Two rabbits were given 0.1 c.c., and two were given 0.2 c.c. of the toxin-sulfapyridine mixture. A similar concentration of toxin in saline was given each rabbit for the control. There was some variation in the size of the necrosis in the same rabbit where the toxin was injected and also where the toxin-sulfapyridine mixture was given. The necrosis was smaller in some of the areas where sulfapyridine and toxin were injected than it was where only the toxin was given. This variation is not significant, however, since there is a variation in the size of the skin necrosis when the same amount

of toxin is given to a single rabbit. Fig. 3 shows the necrosis produced by 0.1 c.c. of staphylococcus toxin given intradermally at A, and the necrosis produced by a similar amount of staphylococcus toxin and sulfapyridine at B.

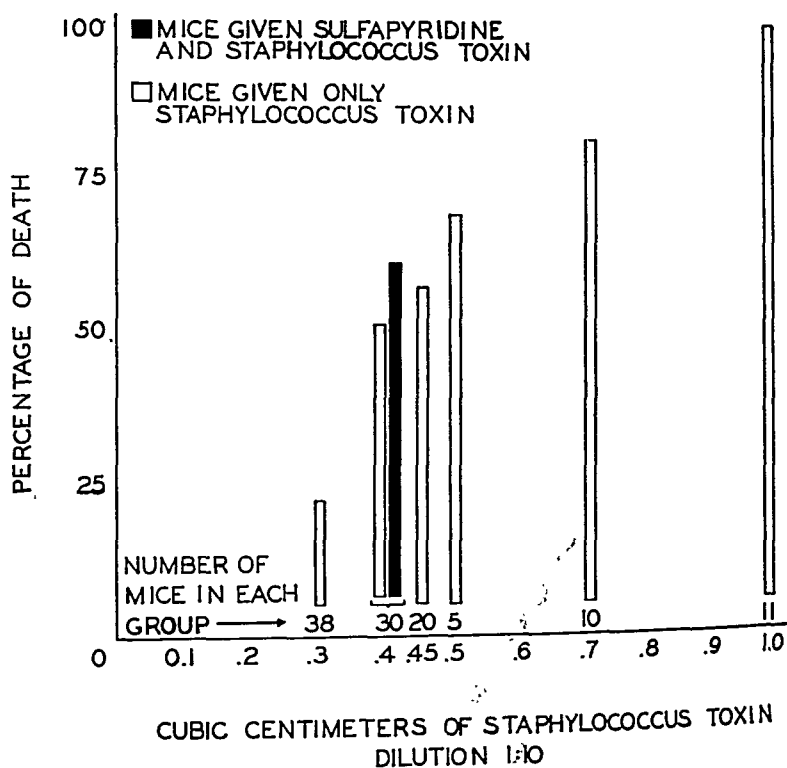


Chart 2.—Percentage of death in mice given staphylococcus toxin intraperitoneally and also staphylococcus toxin containing 20 mg. of sulfapyridine per cubic centimeter.

#### EFFECT OF SULFAPYRIDINE ON LETHAL FACTOR IN STAPHYLOCOCCUS TOXIN

Normal white mice were used in this study. The toxin was given intraperitoneally, and the animals were observed for forty-eight hours. The percentage of death was based upon the number of mice dying during this period. The toxin was diluted 1:10 with saline. Twenty milligrams of sulfapyridine per cubic centimeter were added to this diluted toxin. The toxin-sulfapyridine suspension and the toxin were injected immediately following dilution. The number of mice injected and the per cent of death are shown in Chart 2. It is evident from these data that sulfapyridine in the concentration used, when given at the same time as the toxin, has no inhibitory action on the lethal factor in this toxin.

#### DISCUSSION

It is evident from the data presented in this paper that sulfapyridine extract broth is bacteriostatic for staphylococci. The degree of inhibition in growth apparently is proportional to the concentration of the drug as used in these experiments. This inhibitory action of sulfapyridine on staphylococci apparently can be demonstrated more easily with a small inoculum.

Different strains of staphylococci vary in their susceptibility to the action of sulfapyridine. It is interesting to observe that the strain of staphylococcus most susceptible to the action of sulfapyridine in this experiment is a non-toxin-producing organism. In contrast to this, the two strains of staphylococci most resistant to this drug are both toxin-producing organisms. It will be necessary to make subsequent studies to determine whether there is any correlation between toxin production and the bacteriostatic resistance of staphylococci to sulfapyridine.

The failure of strain "7" to grow in sulfapyridine within a period of seventy-two hours suggests that this drug may be bactericidal. The absence of growth in extract broth in the tubes containing sulfapyridine suggests that certain strains of staphylococci were either killed by this chemical or died spontaneously during the interval.

The appearance of a granular type of growth in the tubes containing sulfapyridine after forty-eight to seventy-two hours might suggest that the bacteria were clumped as a result of the action of sulfapyridine. The diminution in growth of the staphylococci parallels the development of these granules. These observations indicate that there is a variation in the susceptibility of the three strains of staphylococci to a similar concentration of sulfapyridine. Keefer and Rantz<sup>4</sup> have also found a considerable variation in the bactericidal effect of sulfanilamide on different serologic types of streptococci. Meyer<sup>5</sup> has described a clumping of hemolytic streptococci in a serum medium containing a sulfanilamide-sugar compound, which is apparently similar to that observed in the present experiments with staphylococci grown in sulfapyridine.

All the experiments in which staphylococcus toxin and sulfapyridine were used do not give any indication that this drug inhibits any of the actions of this toxin. This observation is interesting in view of Carpenter's studies with staphylococcus toxin and sulfanilamide. Carpenter and his associates<sup>6, 7</sup> found that the lethal action of staphylococcus toxin for mice was inhibited when the toxin was mixed with sulfanilamide preceding an intraperitoneal injection. Rigdon and Avery, in unpublished experiments, found that the hemolytic and skin-necrotizing action of staphylococcus toxin was not inhibited by either sulfanilamide or sulfapyridine. The inhibition of hemolysis about colonies of staphylococci growing on blood agar plates in the presence of sulfapyridine is apparently due to the bacteriostatic action of the drug on the organism and not to any specific effect of the drug upon the hemolysin.

#### SUMMARY

Sulfapyridine in extract broth has a bacteriostatic effect upon staphylococci. There is, however, a considerable variation in the action of this drug on different strains of staphylococci. There is some indication from this study that sulfapyridine may be also bactericidal for some strains of staphylococci when grown in vitro.

The hemolytic, the skin-necrotizing, and the lethal factors in staphylococcus toxin apparently are not affected by sulfapyridine.

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## THE ACTION OF DIGITALOID GLUCOSIDES ON THE VASOMOTOR CENTER\*

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**I**N ANIMAL experiments, digitalis and its allies, when injected intravenously, may raise the blood pressure as high as 300 mm. Hg or more. The efficiency of the heart, as shown by Cushny,<sup>1</sup> is insufficient to account for the whole phenomenon. It is assumed that the vasomotor center is involved, and that the rise is due to a constrictor action on the arterioles. The object of this report is to present evidence of a direct action of digitaloid glucosides on the vasomotor center.

*Digitalis Given Intravenously.*—The average fatal dose of digitalis of the strength of the International Standard is 1.2 mg. per kg. weight in the dog, when given at the rate of 0.1 c.c. per kg. every five minutes.<sup>2</sup> The pressure may rise slightly from the beginning, but not significantly, until *about* one-half the toxic dose has been administered, that is, until toxic symptoms begin to be manifest. In most cases the total rise is about 60 mm. Hg. In a smaller percentage of cases the rise may be enormous. This rise, occurring only in the toxic stage, suggests a central action, since the heart at this time is becoming inefficient.

Although digitalis injected intravenously may cause at first a sharp, temporary fall of blood pressure, this does not occur with proper administration. A fall of pressure is due to too rapid injection, injection shock, which may be elicited by almost any substance injected rapidly. In the present work, we present evidence to show the direct action of digitalis on the centers in the nervous system.

*Method.*—Dogs are anesthetized with intraperitoneal injections of 35 mg. of pentobarbital per kilogram body weight. To show central action the dig-

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italoid drug is injected directly into the fourth ventricle. A continuous record of the blood pressure before and after the injection is made.

Injection is easily done by inserting a hypodermic needle into the region, making the puncture about 1 inch to  $1\frac{1}{2}$  inches below the occipital protuberance, with the head bent sharply toward the chest. The flow of the clear fluid is proof of the location. Following such puncture, however, the blood pressure returns rather rapidly to the normal level (Fig. 1).

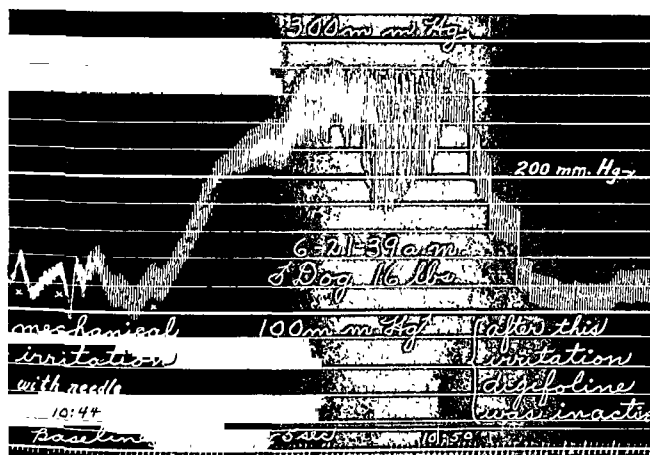


Fig. 1.—Rapid rise in blood pressure induced by mechanical irritation of floor of fourth ventricle by hypodermic needle.

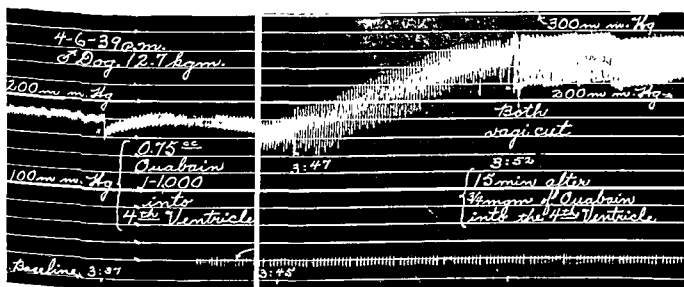


Fig. 2.—Rise in blood pressure effected by 0.75 mg. ouabain injected into the fourth ventricle.

One may occasionally irritate the vasomotor center or tract and immediately raise the pressure to enormous heights (300 mm. Hg). After such injuries digitalis may be inactive centrally. The rise due to digitaloid drugs has a slower onset and persists much longer.

*Effect of Digitaloid Drugs Is Not Due to Irritant Action.*—It is necessary to rule out local irritation. It is well known that an irritant action readily occurs if hypertonic salt solutions are injected into this region. We believe irritation by hypotonicity or hypertonicity has been ruled out by the slow effect in many cases, and also by the absence of irritative reactions, such as muscular twitching, or immediate change in blood pressure.

*Action of Ouabain.*—We commenced with ouabain because of the very dilute solutions needed, and because it furnished a clear solution comparable with the cerebrospinal fluid. Fig. 2 shows the typical effect of ouabain.

*Digifoline N.N.R.*—Sterilized digifoline contains no alcohol or glycerin, and is a clear solution containing the active ingredients of 0.1 Gm. digitalis leaf in 2 c.c. Fig. 3 shows the effect of this preparation.

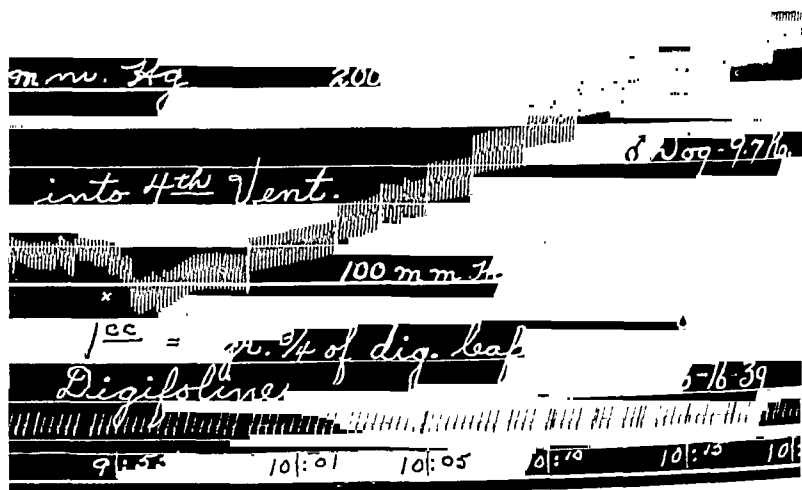


Fig. 3.—Change in blood pressure effected by digifoline injected into the fourth ventricle.

Fig. 4 shows the effect of digifoline evaporated and dissolved in 70 per cent alcohol. Note that the action develops rapidly and persists. Alcohol alone has no such action on blood pressure. It is rather surprising the volume of alcohol that may be injected without affecting the blood pressure or preventing the digitalis action.

That alcohol has little influence, except in hastening the onset of action, due presumably to hastening penetration, is shown by Fig. 5. We have obtained similar effects with an infusion of digitalis.

*Paralysis of the Vasomotor Center by Alcohol.*—One can, with very large doses of alcohol, paralyze the vasomotor center so that digitalis given centrally has no effect. Fig. 6 shows this effect. Note that in this experiment, when relatively large amounts of spinal fluid were replaced with alcohol, the tincture of digitalis centrally caused no rise in blood pressure, but the peripheral action of digitalis was still obtainable.

*Relative Susceptibility of the Vital Centers to Alcohol.*—In our experience in this work the respiratory center is the first to be paralyzed, so that artificial respiration is needed throughout the experiment. The vagus center, as in-

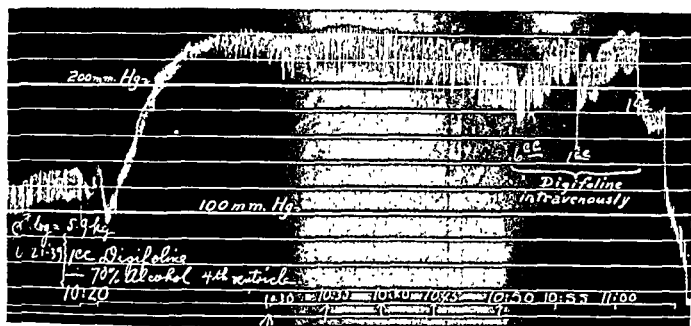


FIG. 4.—Rapid rise in blood pressure effected by digifoline residue dissolved in 70 per cent alcohol. Compare with more gradual rise shown in Fig 3.

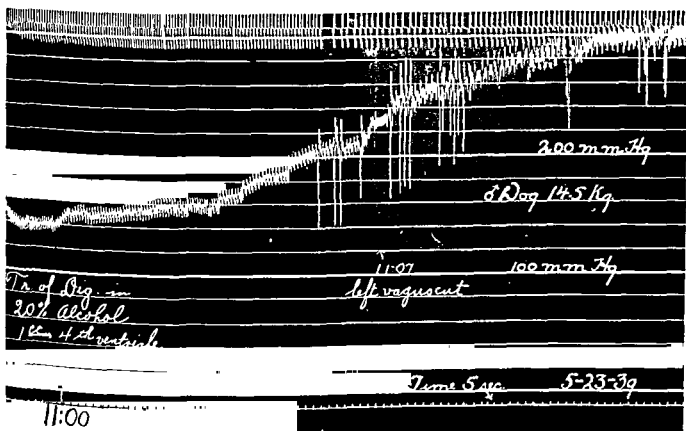
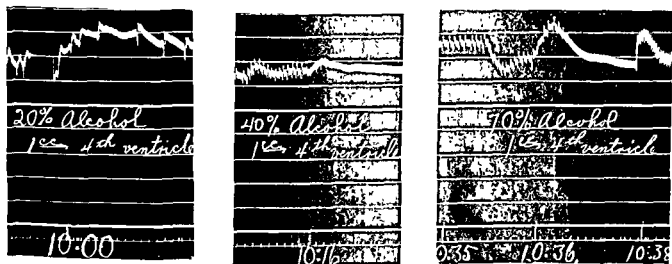


FIG. 5.—Small quantities of alcohol injected into the fourth ventricle have no appreciable effect on the blood pressure, while tincture of digitalis residue suspended in 20 per cent alcohol effects a rise in blood pressure to 310 mm. Hg.

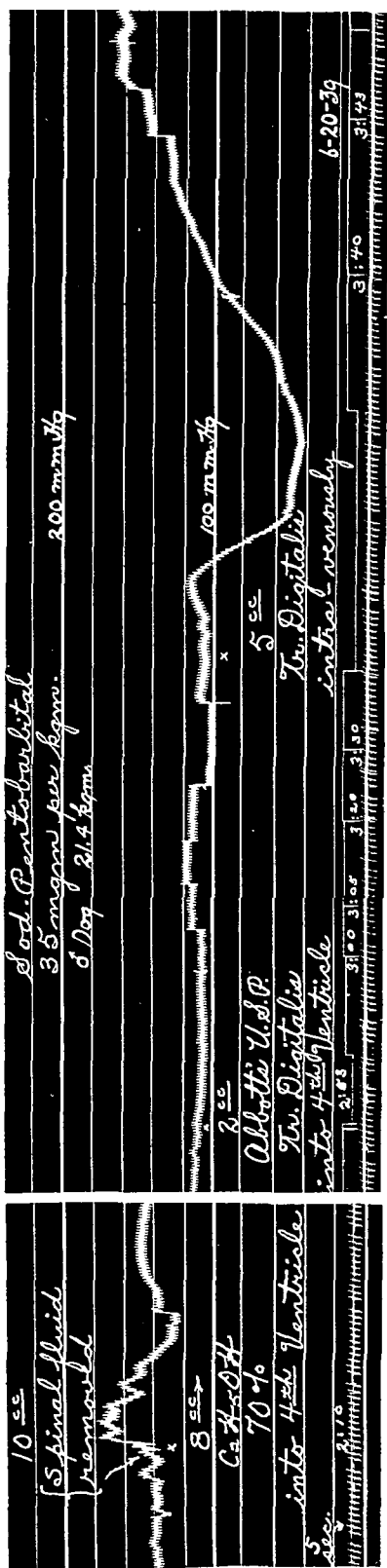


Fig. 6.—A large quantity of 70 per cent alcohol paralyzes the vasomotor center. Tincture of digitalis placed into the fourth ventricle did not effect a rise in blood pressure, but when given intravenously caused an appreciable rise in blood pressure.

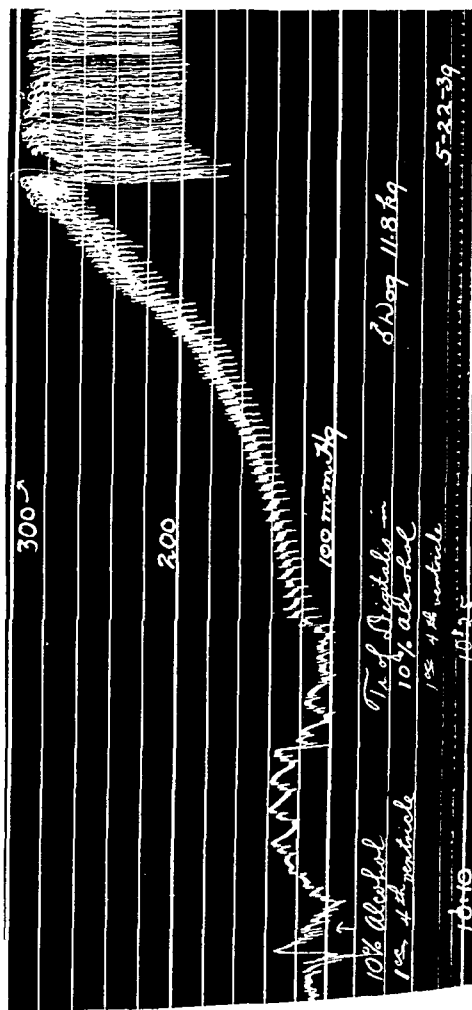


Fig. 7.—With 10 per cent alcohol injected into the fourth ventricle effects but a very slight rise in blood pressure. Tincture of digitalis readily ascertained in 10 per cent alcohol effects a very rapid marked rise in blood pressure.



dictated by the effect on the blood pressure tracings, is the second to be paralyzed, while the vasomotor center is much more resistant.

*Tincture of Digitalis.*—A commercial standard tincture (containing 70 per cent alcohol), which we had tested in the usual way on dogs, was given intravenously. It was necessary first to test the effect of the alcohol. If properly injected, alcohol exerts a depressant action only. Occasionally, however, one may get a pronounced rise of pressure. Indeed one may get a rise to 300 mm. Hg in this way, which is not due to alcohol but to direct stimulation with the needle. It is necessary, therefore, to rule out puncture stimulation. One may inject several times the volume of alcohol (3 c.c.) used in the tincture of digitalis (1 c.c.) without preventing the digitalis rise in pressure. Such doses of alcohol usually paralyze the respiratory center so that artificial respiration is necessary. By injecting larger volumes of alcohol, one may depress the center to such a degree that digitalis following the alcohol is inert. Fig. 7 shows the typical effect of a solution of digitalis in dilute alcohol injected into the fourth ventricle.

#### SUMMARY AND CONCLUSIONS

The central action of digitaloid glucosides in raising the blood pressure is shown. That there is also a peripheral factor involved is also indicated. After the central action has reached its maximum in many cases an added rise may be obtained by injecting the drug intravenously.

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### QUANTITATIVE PROTHROMBIN AND HIPPURIC ACID DETERMINATIONS AS SENSITIVE REFLECTORS OF LIVER DAMAGE IN HUMAN SUBJECTS\*

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WITH the development of quantitative methods for the determination of plasma prothrombin by Quick,<sup>1</sup> and Warner, Brinkhous, and Smith,<sup>2, 3</sup> the liver was established as an extremely important intermediary in the production of this coagulation element. Various investigators have reported a decrease in prothrombin associated with liver damage, but no attempt has been made to correlate the level of the plasma prothrombin with varying degrees of hepatic damage or with the results of the various common liver function tests.<sup>4</sup> Smith, Warner, and Brinkhous reported a marked decrease in prothrombin after severe chloroform-induced liver damage in dogs. The extirpation of 60 to 75 per cent of the liver in rats<sup>5</sup> resulted in a temporary decrease of 30 to

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40 per cent in plasma prothrombin, with a subsequent return to normal during the ten days to three weeks required for the regeneration of the liver to its normal weight. Scanlon, Brinkhous, Warner, Smith, and Flynn<sup>6</sup> failed to produce an increase in plasma prothrombin with bile salts and vitamin K in an individual with cirrhosis of the liver. Stewart<sup>7</sup> noted that when hypoprothrombinemia occurred with obstructive jaundice, the more severe the liver damage the less marked was the prothrombin recovery with therapy consisting of bile salts and vitamin K.

It has been shown that there is no apparent correlation or relationship between fibrinogen and prothrombin.<sup>3, 7</sup> In the present study, observations have been made on normal human subjects and on patients with various degrees of liver damage without biliary obstruction or fistulas. The two-step method of Warner, Brinkhous, and Smith was used to determine the level of the plasma prothrombin. The prothrombin, expressed in per cent of normal, was then compared with the plasma fibrinogen and the galactose, bromsulphalein, and Quick hippuric acid function tests.<sup>8</sup>

The one-step methods of Quick,<sup>1</sup> and Smith, Ziffren, Owen, and Hoffman<sup>9</sup> do not measure the prothrombin quantitatively in every instance<sup>10</sup>; therefore they were not used in this study. This is particularly true in some patients with hypoprothrombinemia, who have developed a rapid conversion rate of prothrombin to thrombin as a compensatory mechanism. In such instances, a prothrombin level of 30 to 40 per cent of normal, as determined by the two-stage method, may give a reading of 80 to 100 per cent of normal by methods which measure in one step the rate of conversion of prothrombin to thrombin and the interaction of thrombin and fibrinogen to form the fibrin clot.

#### OBSERVATIONS

In a series of 41 patients without obstructive jaundice or biliary fistulas the quantitative level of the plasma prothrombin was found to correlate closely with the quantity of hippuric acid excreted (Fig. 1). In addition to normal individuals the series included persons with cirrhosis of the liver, Banti's syndrome, familial hemolytic icterus, pernicious anemia, aplastic anemia, multiple myeloma, polycythemia vera, Hodgkin's disease, and leucemia of all types with various degrees of liver infiltration. In those individuals with proved liver damage in which the hippuric acid excretion was 0.86 to 2.0 Gm., the prothrombin was found to be 19 to 37 per cent of normal; with 2.0 to 3.9 Gm. of hippuric acid excretion, the prothrombin level was 33 to 90 per cent of normal; and with 3.9 to 4.56 Gm. hippuric acid excretion, the prothrombin level was 70 to 100 per cent of normal.

There was no consistent correlation between plasma prothrombin and plasma fibrinogen, particularly when an infectious process was present. In one patient with aplastic anemia, oral sepsis, continuous high fever of 104° F., and a resulting acute hepatic damage, who was given daily transfusions, the prothrombin was only 30 per cent of normal, but the fibrinogen was elevated to 0.531 Gm. per cent. The icterus index was 24 units. On admission the prothrombin was 94 per cent of normal, and the fibrinogen was 0.483 Gm. per cent. In another individual with aplastic anemia the admission prothrombin

was 69 per cent of normal, the fibrinogen 0.321 Gm. per cent, and the icterus index 6 units. This patient subsequently developed an infection in the gluteal region, sustained a high elevation in temperature, became jaundiced (icterus index 78), the fibrinogen became elevated to 0.518 Gm. per cent, but the prothrombin decreased to 24 per cent of normal.

The galactose tolerance test was within normal limits in all patients studied. There was a wide variation in the results recorded for the bromsulphalein dye test.

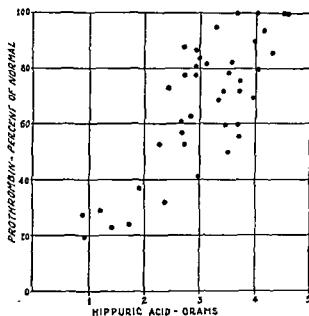


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acid output was always accompanied by a pro-  
thrombin level in the blood. Each point represents a different patient

The following two cases of advanced hepatic cirrhosis illustrate the variation in the different liver function tests and the levels of plasma prothrombin and fibrinogen.

CASE 1 (Fig. 2).—R. W., a white male, aged 13 years, was admitted to the University Hospital with the complaint of frequent attacks of epistaxis and bleeding gums of about three years' duration. Positive physical findings on admission included icteric sclera, hemorrhagic gums, coagulated blood in the nares, and enlarged liver and spleen. A diagnosis was made of hepatic cirrhosis with splenomegaly (Banti's syndrome). The plasma prothrombin was 24 per cent of normal, and the fibrinogen was 0.288 Gm. per cent. Liver function tests: Quick hippuric acid test 1.71 Gm.; bromsulphalein dye retention test (5 mg. per kg. body weight) 10 per cent in one-half hour and 5 per cent in one hour; galactose tolerance test 2.6 Gm. The icterus index was 13.3 units. After a month's observation the patient was given intensive therapy with bile salts and vitamin K (the extract from 200 Gm. of alfalfa daily for a period of three months). There was only a negligible variation in the level of the plasma prothrombin. The patient continued to have occasional attacks of epistaxis and bleeding gums but gained in weight and carried on the normal activities of a boy his age.

CASE 2 (Fig. 3).—J. K., a white female, aged 15 years, vomited a large quantity of blood, necessitating two emergency blood transfusions, the day before admission to University Hospital. She had had previous attacks of what was thought to be catarrhal jaundice, for which sulfanilamide therapy had been prescribed. On physical examination there was a slight icteric color to the sclera and the skin. The liver was tender to palpation. The spleen later became palpable and ascites developed. The prothrombin on admission, in spite of 1,000 c.c. of blood received by transfusion, was only 28 per cent of normal, and the fibrinogen was 0.207 Gm. per cent. The icterus index was 25 units. Liver function tests: Quick hippuric

acid test 1.4 Gm.; galactose tolerance test 3.35 Gm. Bile salts and vitamin K (the extract of 400 Gm. of alfalfa) were given daily for ten days, with no appreciable change in the level of the plasma prothrombin. During an attack of acute parotitis with an elevation in temperature, there was an increase in the fibrinogen with no variation in the prothrombin level. She died following a severe hematemesis. At autopsy the diagnosis was made of toxic cirrhosis with an early attempt at liver cell regeneration, ascites, splenomegaly, and bilateral hydrothorax.

#### DISCUSSION

There is a definite correlation between the level of the plasma prothrombin and the synthesis and excretion of hippuric acid following the ingestion of sodium benzoate, as measured by the method of Quick. Observations confirmed at operation and autopsy have shown that these two tests are sensitive reflectors of liver damage. Other investigators have reported that the hippuric acid test is a good index of the amount of hepatic damage present.<sup>11-13</sup> The observations of Warner<sup>5</sup> with partial extirpation of the liver in rats and the resulting hypoprothrombinemia show that there is a definite relationship between the amount of liver damage and the level of the plasma prothrombin. Vitamin K and bile salts have no effect on the level of the prothrombin when the hypoprothrombinemia is due to extensive injury to the parenchymatous tissue of the liver. A decrease in prothrombin in patients without obstructive jaundice or biliary fistulas may not be due to lack of vitamin K or to any decrease in the absorption from the intestinal tract.<sup>6</sup> In our experience individuals with mild chronic pathologic states frequently show a decrease of 10 to 25 per cent in the level of the plasma prothrombin.

The methods most widely used at the present time for the quantitative determination of prothrombin are the two-stage titration technique of Warner, Brinkhous, and Smith,<sup>2, 3</sup> and the one-stage techniques of Quick,<sup>1</sup> and Smith, Ziffren, Owen, and Hoffman.<sup>9</sup> The two-stage titration technique is undoubtedly the more accurate but it is difficult to use as a routine laboratory procedure. In many instances, however, the one-step method does not measure the quantity of prothrombin because it is an index of many variable factors, including (1) the amount of prothrombin, (2) the rate of conversion of prothrombin to thrombin, and (3) the reaction of thrombin with fibrinogen to form the fibrin clot. In the two-step method, the prothrombin is first converted to thrombin, and the thrombin is then titrated. There is a wide variation in the conversion rate in various species.<sup>14</sup> The ease with which this factor may vary and the differences in the results of the two methods have been conclusively demonstrated in infants during the first few days of life, in which the prothrombin may actually be very low, but because of the rapid conversion rate normal values are recorded by the one-step methods.<sup>10</sup> In one of our patients (Case 1, R. W.) in whom there was a consistent hypoprothrombinemia secondary to cirrhosis of the liver, the tendency to hemorrhage was variable. At times there was a definite compensatory mechanism in which the conversion rate was very rapid, the actual prothrombin value being 32 per cent of normal as measured by the two-step method, and 80 per cent of normal by the one-step method. Inasmuch as the one-step technique in some instances does not accurately determine the amount of prothrombin,

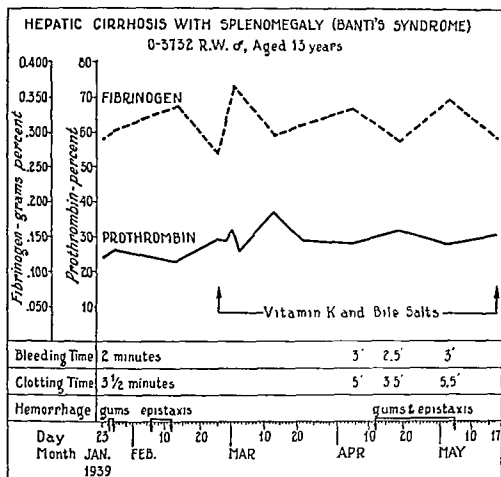


Fig. 2.—Illustrating the lack of response of the plasma prothrombin to seventy-nine days' intensive therapy with vitamin K and bile salts in an individual with advanced hepatic cirrhosis and splenomegaly (Banti's syndrome). The fibrinogen remained consistently within normal limits. The Quick hippuric acid test was 1.71 Gm.; galactose tolerance test 2.6 Gm.; and the bromsulphalein dye retention test 10 per cent in one-half hour, 5 per cent in one hour.

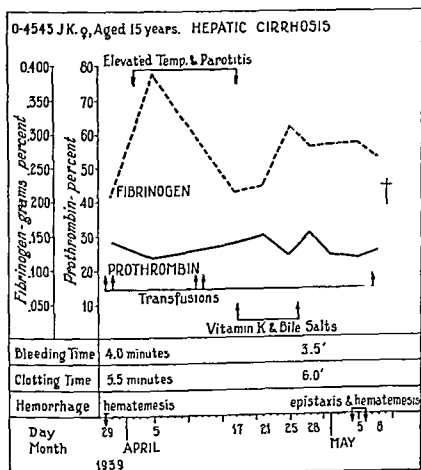


Fig. 3.—Illustrating the lack of response of the plasma prothrombin to intensive therapy with vitamin K and bile salts in an individual with a rise in the plasma fibrinogen during an attack of acute parotitis. Transfusions had very slight effect on the level of

intensive  
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being nevertheless an excellent practical measure of the tendency to hemorrhage, the results of such tests cannot be used as indices of liver function.

There is no consistent correlation between the levels of the prothrombin and fibrinogen in the plasma. Smith, Warner, and Brinkhous<sup>3</sup> reported that the prothrombin formation is more easily disturbed than fibrinogen formation, and observed an increase in fibrinogen with a sustained low prothrombin level during an episode of canine distemper which complicated the recovery of one of their dogs following chloroform-induced hepatic damage. In the two patients already cited, who developed a sustained high temperature secondary to infection and an acute toxic hepatitis, the same phenomenon was observed. It has been shown that uncomplicated fever induced by the Kettering hypertherm may produce liver damage with a resulting hypoprothrombinemia but that the fibrinogen may not be affected.<sup>15</sup> Ham and Curtis<sup>16</sup> observed only slight variations in the fibrinogen level when hyperpyrexia was induced by high environmental temperature, whereas following intravenous injections of typhoid vaccine, the level rose sharply. The level of the plasma prothrombin may be decreased if there is liver damage due to any cause, but there is no stimulation to an increased overproduction with concurrent infections. Stewart<sup>7</sup> emphasized the lack of correlation between the quantitative prothrombin and the fibrinogen determinations. It would seem that the level of the fibrinogen is an extremely poor index of the relative degree of hepatic damage.

A relatively extensive degree of damage to the parenchymatous tissue of the liver must be present before it is reflected in many of the commonly used liver function tests. This is easily understood as Mann<sup>17</sup> has shown that many of the physiologic functions of the liver may be maintained at normal levels with only a very small portion of the liver intact, or after generalized damage to the entire parenchymatous tissue. Apparently the ability of the liver to form prothrombin from vitamin K and to synthesize hippuric acid from sodium benzoate is a more sensitive measure of the amount of liver damage present than the majority of the liver function tests.

It is interesting to note that prothrombin cannot be replaced by blood transfusion in sufficient quantity to be of any therapeutic value. The changes in the total content of prothrombin in the plasma of the recipient are dependent on the prothrombin content of the plasma of the donor, and may be calculated on the basis of the direct additive<sup>18</sup> effect. Theoretically, if a transfusion of 500 c.c. of blood containing the normal quantity of prothrombin is given to a person weighing 70 kg., the level of the plasma prothrombin would be elevated only 7 per cent. In one of the cases of aplastic anemia with a prothrombin of 30 per cent of normal, daily transfusions were given without controlling the hemorrhagic diathesis produced by a combined hypoprothrombinemia and thrombocytopenia. In another patient with a pretransfusion level of 23 per cent of normal, the prothrombin was 28 per cent of normal after two transfusions of 500 c.c. each. Stewart<sup>7</sup> observed that blood transfusions appeared to be a rather inefficient method of combating the bleeding tendency due to hypoprothrombinemia, as the effect on the recipient's

plasma prothrombin is slight and transitory, and reported an increase of only 6 per cent in one adult patient whose plasma prothrombin was determined before and after transfusion of 600 c.c. of blood.

#### CONCLUSIONS

1. In the patients here studied, without biliary obstruction or biliary fistulas, the quantitative levels of plasma prothrombin and the amounts of hippuric acid excreted following the ingestion of a known quantity of sodium benzoate (Quick test) would seem to have reflected most sensitively and consistently the existing degree of liver damage. There was no such suggestive correlation observed between these tests and the plasma fibrinogen levels, the bromsulphalein dye clearance or galactose utilization, either singly or collectively, when all were studied in the same patient.

2. The one-step methods of Quick, and Smith, Ziffren, Owen, and Hoffman do not accurately determine the true quantity of prothrombin in the blood plasma of some individuals because of the variability in the rate of its conversion to thrombin; hence in some instances the results of the one-step techniques, though they are an index of the tendency to bleed, cannot be correlated with variations in liver function.

3. Transfusions are of little or no therapeutic value in the control of the hemorrhagic tendency in individuals with hypoprothrombinemia.

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# A REVIEW OF ADVANCES IN THE STUDY OF AURICULAR DISORDERS\*

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CLINICAL research on disorders of the auricles contribute to our knowledge of the physiology and pathology of the heart. In man there is need to study the possibility of physiologic pathways through the auricular myocardium, the constant presence of which Eyster and Meek, Rothberger and his associates, and Condorelli have demonstrated in laboratory animals by means of ligatures.

Some pathways lead the stimulus directly from the sinoauricular node to the right auricle (superior sinoauricular bundle, inferior sinoauricular bundle, and anterior sinoauricular fibers). An important pathway (the Bachmann or interauricular bundle), among others, carries the stimulus from the sinoauricular node to the left auricle. Three principal pathways, as Condorelli has shown, carry the stimulus from the sinoauricular node to the auriculoventricular node: (1) the inferior sinoauricular nodal, or Wenckebach bundle, arises in the tail of the sinoauricular node and passes along the torus lowerii between the venae cavae to the interauricular septum; (2) the superior sinoauricular septal, or Condorelli bundle, accompanies for some distance the Bachmann bundle, then turns downward, penetrating the septum; (3) some fibers arise in the posterior part of the sinoauricular node, go around the superior vena cava, and then penetrate the septum. Before entering the auriculoventricular node, these three principal pathways meet at the Condorelli crossing point.

These pathways are not anatomically differentiated bundles in the sense of the bundle of His. Some represent only the shortest route, and, therefore, the pathway of impulse conduction; others seem to be functionally differentiated. When some of these pathways are mechanically interrupted, the stimulus follows a longer course, and changes in the shape of the electrical wave occur. The Condorelli crossing point, however, is exceptional in that ligation here produces complete auriculoventricular dissociation. Although the three classical electrocardiographic leads have thrown much light on auricular disorders, special leads have been devised for attempting to record separately and simultaneously the electrical waves in each auricle, trying to secure more exact and important information.

## METHODS

In 1910 Lewis<sup>1</sup> observed that placing the electrodes on the chest wall in the vicinity of the right auricle caused the small and frequent oscillations given by patients with auricular fibrillation to be much higher and more distinct.

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In 1924 Ackermann<sup>2</sup> began a new search for such special leads, describing a method by which two needles were inserted under the skin in the second and fifth interspaces near the right sternal border. He observed that P waves taken in this way were particularly high.

Condorelli<sup>3</sup> in 1931 very carefully studied a patient with disordered auricular action, using a slight modification of Ackermann's method; the auricular waves, produced, he thought, by the right auricle (but actually in all probability by both auricles), were best recorded with the needles in the second and fourth interspaces. A new lead, with one needle in the third interspace at the left sternal margin and the other near the fifth dorsal vertebra on the left paravertebral line, gave a record of auricular waves which he thought might represent left auricular activity. Laufer,<sup>4, 5</sup> working in my laboratory, applied Condorelli's leads to further patients, and Lian, Merklen, and Odinet<sup>6</sup> described an anterior lead for the right auricle and a posterior lead for the left auricle.

Lieberson and Lieberson<sup>7</sup> in 1934 introduced their technique, which was an important advance, with one electrode in the esophagus and the other (the so-called "indifferent electrode") on the left leg.

In 1935 I<sup>8</sup> made a further advance in developing this new method of auricular registration: a thin bipolar sound placed in the esophagus near the left auricle recorded the waves of this auricle; another record of auricular action was made by means of two plates, one applied to the tongue and the other over the xiphoid process, to represent prevalently right auricular action.

After careful study of fourteen different combinations of leads on the same patient, the esophageal method was chosen because its aim of securing a differentiation from the right or combined auricular P wave (P right) of the left (P left) seemed a possibility for the following reasons:

1. Higher voltage of the left auricular wave as compared with the voltage of the ventricular complexes.
2. Shorter duration of the left auricular wave.
3. Shorter P-R interval.

The longer P-R interval and the higher voltage of the right auricular wave were the reasons that the mouth-xiphoid method was chosen. By this technique the registration of waves from what was thought to be the right auricle appeared as good as in Condorelli's method, while the left auricular waves were more satisfactory than in previous methods.

Laufer and Rubino<sup>9-11</sup> and Sossai<sup>12</sup> applied this technique systematically to all patients with cardiac disturbances in my laboratory for several years. Later, Brown,<sup>13</sup> Zårday,<sup>14</sup> and recently Deglaude and Laubry,<sup>15</sup> and Spühler<sup>16</sup> applied the unipolar esophageal method to many patients with auricular disorders.

In 1937 de Chatel and Hussey<sup>17</sup> in Rothberger's laboratory recognized the possible clinical value of the bipolar esophageal method after applying it to the dog, but were uncertain of its preference to the unipolar method.

Sossai<sup>12</sup> made this clear, however, the following year from a comparative study of the two techniques on many patients with differing rhythms in the two auricles or with delayed conduction to the left auricle, disorders which will be explained more fully later.

With one electrode on a distant part of the body, the recorded P waves represent the activity in both auricles, even though the other electrode is placed near the left auricle. To record the activity in the two auricles separately, therefore, the so-called "indifferent electrode" cannot be used.\* This is in accord with the recent conclusion of Kossmann and Rader.<sup>19</sup>

In man, records of activity in the proximal part of the left auricle by the bipolar esophageal method are usually excellent, and, as a rule, show no clear evidence of the more distant parts, including right auricular activity. When left ventricular hypertrophy is present, as in aortic regurgitation, small ventricular waves may appear. Registration of right auricular activity is less satisfactory because high ventricular waves are seen, showing that the currents of all the heart muscle are recorded; when there is marked delay in contraction of the left auricle, small inverted deflections mark the activity of the left auricle; they are, however, smaller than right or combined auricular waves.

The best comparative method is obtained by simultaneous records through the use of a double-string galvanometer. When this is impossible, it is necessary to take a mechanical record (e.g., a carotid pulse) with each electrocardiogram and to measure the interval from the beginning of the P waves to the beginning of the pulse wave, because in the esophageal lead the R wave is usually small and obscure. The interval between "P right" wave and "P left" wave can easily be measured on the records taken by means of the double-string galvanometer. Working with a single-string galvanometer and a mechanical record, this interauricular interval equals ("P right" wave  $\rightarrow$  pulse) - ("P left" wave  $\rightarrow$  pulse). In the new leads usually the shorter P-R interval is in the esophageal lead, because the excitation of the left auricle physiologically occurs slightly later than that of the right.

The proximity of the electrodes to the auricular musculature makes it almost certain that normally the entire electrical activity is recorded and that no isoelectric phase exists. When the left auricle is much enlarged, however, it is theoretically possible that the distance of the electrodes from parts of the auricle and the changing axis of the auricle relative to the electrodes produce an initial isoelectric phase of the "left P" wave. This would lessen the significance of a recorded delay in contraction of the left auricle.

The normal interval between the beginning of the "P right" wave and the beginning of the "P left" wave is so brief (0.01 to 0.02 second) that the various methods for registering auricular activity must be evaluated on subjects with abnormally long intervals or auricular dissociation.

\*In experiments on the sinoauricular electrogram it is found that when one needle is placed in the sinus region and the other on the right foreleg, it is impossible to obtain a wave representing the sinus activity that is as short and early as that obtained by means of two needles placed in the sinus region.

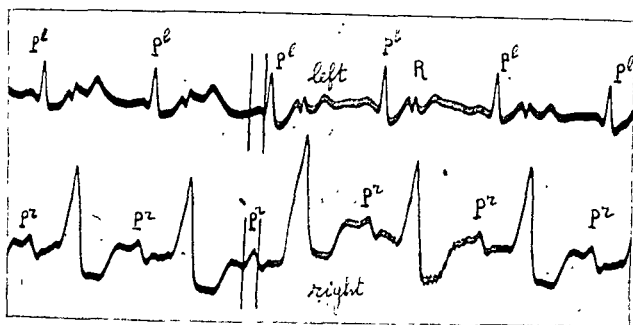


Fig. 1.—Delay of contraction of the left auricle in a patient with aortic regurgitation and coronary heart disease. Upper curve, esophageal lead for the left auricle. Lower curve, lead for the right auricle. (From Luisada.<sup>4</sup>)

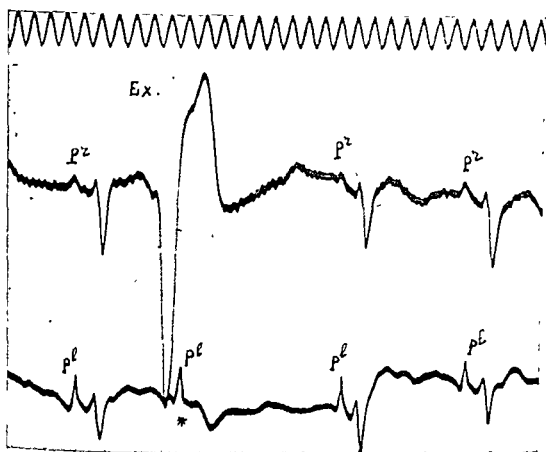


Fig. 2.—Ventricular premature beat. The esophageal lead permits the recording of the P left wave even during the premature beat (lower curve). (From Luisada.<sup>5</sup>)

Comparing these new leads with the three classical leads, Laufer<sup>4</sup> found that the P wave is often wider in the latter. He found that the longer P-R interval recorded in the peripheral leads (that of the second lead) usually is equal to the P-R interval in the new lead for the right auricle. Differences in the P-R interval in the three classical leads, then, are probably due to the better or worse recording of the currents given from the two auricles in the different leads. Normally the P waves of the two auricles are not absolutely simultaneous, and in pathologic conditions they may be further separated.

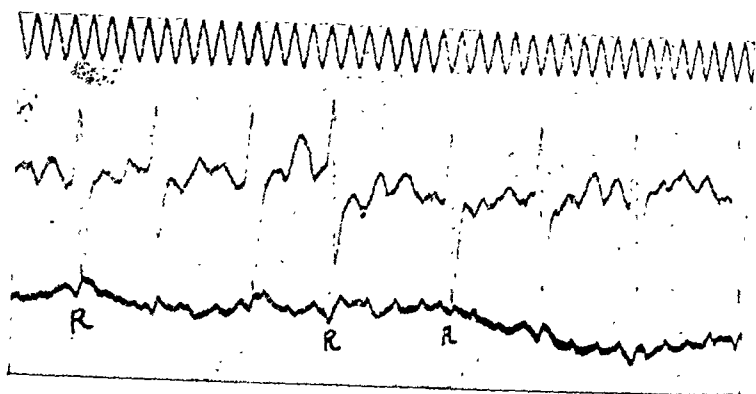


Fig. 3.—Flutter-fibrillation of both auricles. Upper curve, lead for the right auricle. Lower curve, esophageal lead for the left auricle. According to the circus movement hypothesis, the waves could be coarser in the right auricular curve because they are nearer to the circus (From Rubino.<sup>10</sup>)

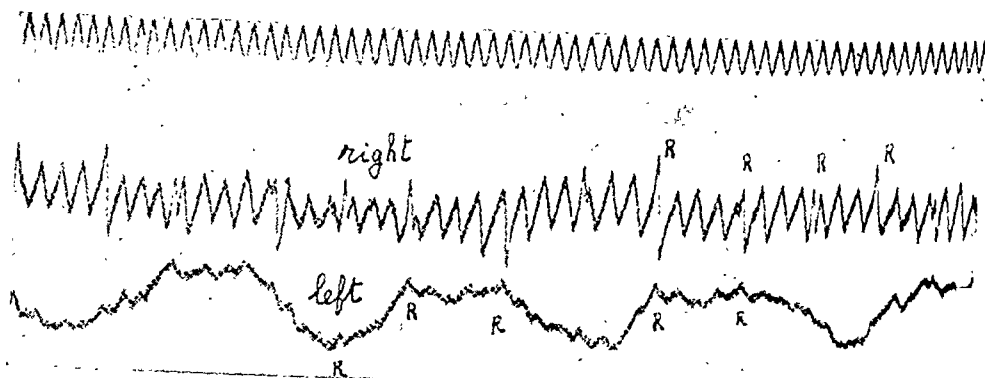


Fig. 4.—Flutter of the right auricle (upper curve), very small, irregular waves (?? fibrillation) in the left auricle (lower curve). It is possible that there is only one circus movement in the right auricle, the deflections in the left auricular curve being due to irregular radiations (From Rubino.<sup>10</sup>)

#### CLINICAL INVESTIGATION OF AURICULAR DISORDERS

Scherf and Shookoff, Rothberger and Scherf, Condorelli, Scherf, and Siedeck, and others have studied intensively experimental disorders of the auricles; Groedel, Dressler, Mahaim, Condorelli, and many more have investigated clinical auricular disorders by means of the classical electrocardiographic leads. The new techniques already described have given a new stimulus for the study of the problems of the following possible auricular disorders:

1. *Delay in contraction of the left auricle.* Condorelli,<sup>3</sup> Laufer and Rubino,<sup>3</sup> Sossai,<sup>12</sup> and Luisada<sup>10</sup> have repeatedly observed a delay of the left auricular contraction in hearts with enlargement of the left auricle in mitral disease, or with myocardial fibrosis due to coronary atherosclerosis or syphilitic atresia of the coronary mouths. The delayed P wave arising from the left auricle may be either of normal height and form, or very small—as Rubino<sup>10</sup> and Sossai<sup>12</sup> have each described in a patient just after the change from auricular fibrillation to sinus rhythm. In cases with prolonged conduction from the sinoauricular node to the left auricle, the classical leads show large, double, or diphasic P waves.

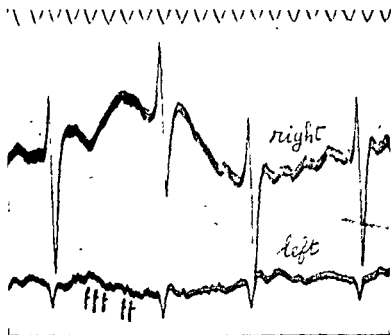


Fig. 5.—Fibrillation of both auricles. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. (From Luisada.<sup>20</sup>)

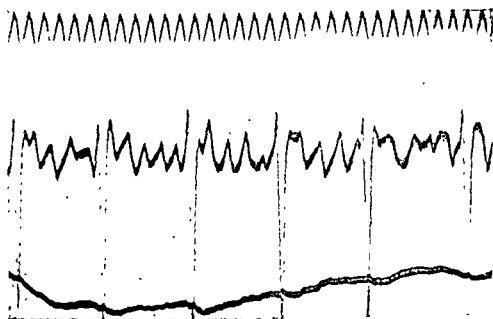


Fig. 6.—Flutter of the right auricle (upper curve) with complete absence of intrinsic electric waves in the left (lower curve). (?? standstill of the left auricle) (From Luisada.<sup>20</sup>)

We have already mentioned a possible purely technical explanation for this delay. The elongation of the muscle tracts in cases with great dilatation of the left auricle may also be a factor.

2. *Delay in contraction of the right auricle.* Rubino<sup>11</sup> and Luisada<sup>19</sup> have considered the possibility of this mechanism during ocular compression in a patient with permanent lengthening of the P-R interval. During the compression the "P right" wave was progressively delayed until it followed the "P left" wave (see Fig. 9).

The occurrence of auricular premature beats without change either of the ventricular rhythm or of the "P left"-R interval is another possible interpretation of these curves. Laufer and Rubino<sup>9</sup> observed also some cases in which the superimposition of the "P waves of the two auricles" suggested simultaneous contraction of the auricles. In such cases one may conjecture that the sino-auricular—right auricle conduction is so delayed that the excitation of the right auricle occurs only when the sinus impulse has already reached the left auricle.

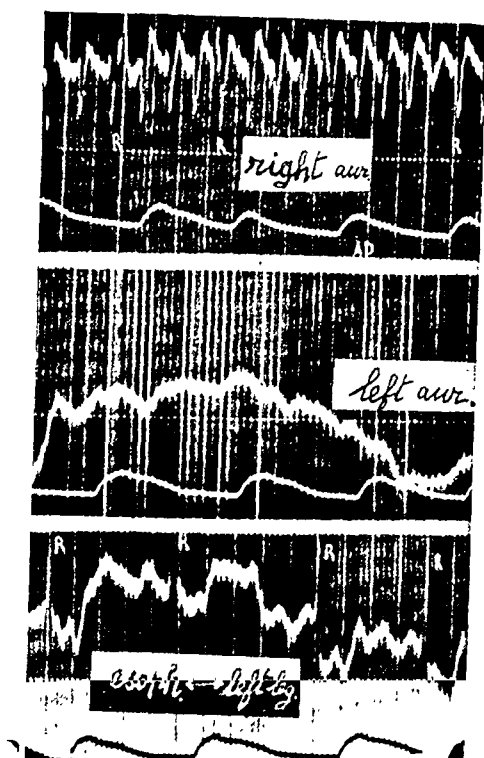


Fig. 7.—Flutter of the right auricle with fibrillation of the left. A, Lead for the right auricle (two-needle method) and brachial pulse (lower tracing). B, Esophageal lead for the left auricle and brachial pulse (lower tracing). C, Esophagus and left leg lead (it records the waves of both auricles). (From Sossai.<sup>12</sup>)

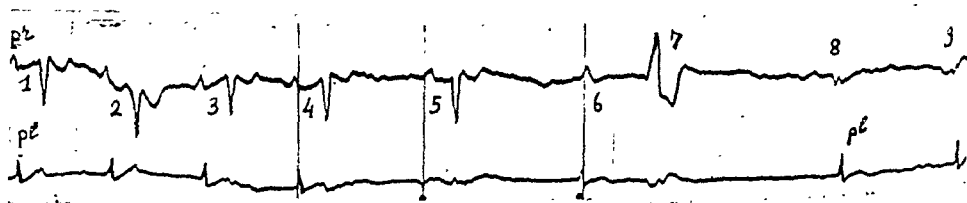


Fig. 8.—Effects of ocular compression in a vagotonic patient. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. The first four complexes are normal. 5 is an ectopic beat with inverted P left wave which precedes the P right wave. 6 is a sinus impulse reaching the left auricle and then the right (delayed conduction to the right auricle, block of impulse to the ventricles). 8 and 9 are two sinus discharges with normal P left waves, but with deformed P right waves and no ventricular complexes. (From Rubino.<sup>11</sup>)

3. *Delay in contraction of both auricles with normal sinoauricular-atrioventricular node conduction and resulting decrease in P-R interval.* Condorelli<sup>11</sup> first established this conception experimentally by means of ligatures in the auricular muscle; later he, Laufer and Rubino,<sup>9</sup> and I<sup>20</sup> thought we found it clinically. Since, however, the electrocardiograph cannot register in man the moment of the sinus discharge, the clinical occurrence of this phenomenon cannot be proved. Two observations support the likelihood of this delayed conduction through the sinoauricular-auricular bundles when the sinoauricular-atrioventricular node conduction is normal or less delayed: first, the shortened P-R interval seen in patients with diffuse myocardial fibrosis with or without

bundle branch block; and second, the shortening of the P-R interval in a vagotonic subject during ocular compression.\*

4. *Conduction block to ventricles and left auricle.* Rubino<sup>11</sup> referred to this possibility after recording esophageal records in a vagotonic patient during compression of the eyeballs. As indicated by the premature appearance of a slightly deformed auricular P wave, an ectopic beat arose in the right auricle, but no P wave of the left auricle and no ventricular complex followed. Artifacts can probably be excluded by the firm placing of the esophageal sound and by the reappearance of normal "P left" waves after several cardiac contractions.

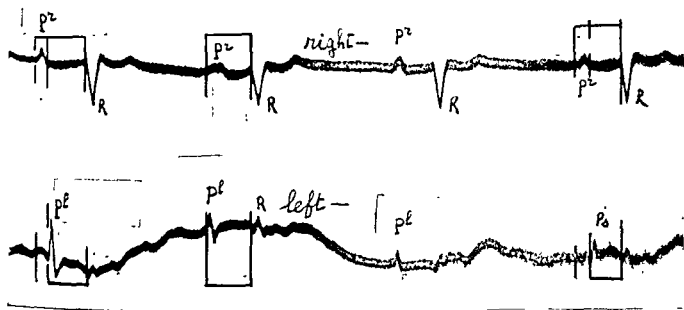


Fig. 9.—Effect of ocular compression in a vagotonic patient. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. The first complex shows a normal auricular and ventricular sequence (P right wave, P left wave, normal P-R interval). The second complex shows deformed synchronous P waves from both auricles and normal P left-R interval (slower conduction to the right auricle). The fourth complex shows deformed P waves with normal succession P right-P left, but shortened P-R interval (apparently slower conduction to both auricles with normal sinoauricular node-auriculoventricular conduction). (From Luisada.<sup>20</sup>)

5. *Auricular premature beats without ventricular contraction.* Rubino<sup>11</sup> observed this in the same patient (in 4 above). Following total cardiac standstill induced by ocular compression, there occurred a series of "P left" waves, preceded, accompanied, or followed by "P right" waves, with no evidence of ventricular activity. When the "P left" waves fell earlier, or the complexes were deformed (or both occurred), it seems probable that the auricular complex was of ectopic origin. The oculocardiac reflex blocked the conduction of sinoauricular impulses to the ventricles.

6. *Flutter of the right auricle with fibrillation of the left.* Open chest experiments on dogs have demonstrated the possibility of dissociated rhythms in the two auricles. By ligating coronary vessels, Condorelli<sup>21</sup> has produced fibrillation of the left auricle while the right auricle beats normally.

Scherf and Siedeck,<sup>27</sup> by ligating muscle bundles, have produced many interesting disorders: (a) independent rhythms in the two auricles, either (1) a slow regular rhythm of the right auricle under the probable control of the

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A-V node, with normal beating of the left auricle under the control of the sinoauricular node; or (2) the reverse of this, with sinus control of the right auricle and probable A-V nodal control of the slower left auricle; (b) fibrillation in the left auricle while the right auricle beats normally; (c) fibrillation in the right auricle while the left auricle beats forcibly but irregularly.

Many dissociated auricular rhythms have been described in man. So far, however, only flutter of the right auricle with either fibrillation or standstill of the left has been apparently indicated by the new techniques.

Laufer,<sup>4</sup> and later Laufer and Rubino,<sup>9</sup> Rubino,<sup>10</sup> Sossai,<sup>12</sup> and Luisada<sup>20</sup> repeatedly reported this interesting disorder.\* In it, the "right auricular" curve shows irregular or regular high deflections, while a simultaneous lead from the left auricle shows frequent tiny deflections of the base line or total absence of intrinsic activity. Rubino,<sup>10</sup> and later Sossai,<sup>12</sup> each reported a remarkable case with this disorder, showing, on resumption of sinus rhythm, small, delayed "P left" waves.

So far, this disorder has been recorded nearly exclusively in patients with mitral stenosis in failure, being apparently favored by unequal distention of the two auricles. Sossai's case, for example, showed an aneurysm of the left auricle; when this decreased in volume following digitalization, the normal sinus rhythm resumed. In all these cases the peripheral leads have shown flutter-fibrillation (impure flutter). As with the cases of delay in contraction of the left auricle, these cases provide good opportunity for comparison of methods of auricular registration, for only the best methods can separate the two rhythms.

7. *Fibrillation or flutter of both auricles.* The occurrence of the same rhythm in both auricles is the usual observation; this may be fibrillation, flutter, or the relatively less common flutter-fibrillation (Rubino<sup>10</sup>). As indicated, impure flutter or tremulation, as recorded in classical leads, is caused by flutter in one auricle and fibrillation in the other, as well as by this flutter-fibrillation in both auricles. Fibrillation of both auricles occurs in patients either with mitral valve disease, or with myocardial fibrosis; at times, however, the greatly distended left auricle produces no deflections in the esophageal lead. A true paralysis of the left auricle could explain this, but mechanical methods are insufficiently delicate to prove it. Any auricular activity in the more normal right auricle may be poorly shown in the distended abnormal left auricle, even though the mechanism may be fundamentally the same in both.

#### DISCUSSION

Methods now available for registering auricular activity have added much to the interest of auricular disorders. Records taken with routine leads had suggested some of these, such as the delayed contraction of the left auricle. Other disorders, as dissociation between the auricles, with flutter in the right and fibrillation in the left, were wholly unsuspected, but now seem likely. Future use of the new methods may well reveal further unusual mechanisms. New theories advanced on the basis of open chest experiments may soon find

\*A new case was described recently by Deglaude and Laubry.<sup>15</sup>



clinical confirmation. It is fundamentally important meanwhile to learn whether chest leads can ever show independent auricular action, that is, either right or left, which appears at present unlikely, and also to learn just how much of the total left and right auricular activities is shown by bipolar esophageal leads, still a moot point.

The classical theories dealing with the disturbed physiology causing auricular fibrillation and flutter will probably be revised in the future, in order to explain the dissociated auricular disorders. It is necessary, however, that more data be collected and statistical studies be made before accomplishing such a difficult task. Much remains for investigation; for example, the occurrence of varying rhythms in the two auricles. The cases with small, rapid, irregular deflections discussed by De Castro<sup>22</sup> and by Dagnuni<sup>23</sup> as examples of "partial auricular fibrillation" (although obtained by an inadequate technique) may perhaps fall into this group and should be confirmed by the new methods.

The occurrence of *standstill of both auricles*, demonstrated by White<sup>24</sup> by both electrical and mechanical methods, and since confirmed by others (Levine<sup>25</sup>), has not yet been investigated by means of the new techniques. Neither has the occurrence of *alternate excitation of the right and the left auricle* (Lian and Golblin<sup>26</sup>; Scherf and Siedeck<sup>27</sup>), as suggested in the classical leads.

It must not be concluded from the foregoing observations that the two auricles are completely separate functional entities, although at times they may react independently in their properties of conductivity and contractility. True dissociation of the two auricles occurs only when the usual conduction pathways are interrupted by anatomic blocks, such as Condorelli, Scherf and Siedeck, and others produced in open chest experiments.

#### SUMMARY

1. Methods for the simultaneous and separate study of electrical activity in the auricles are reported.
2. The possibility of various bizarre disorders of auricular action in man, suggested by these methods, is discussed. Such possible disorders are: (a) delay in contraction of one auricle (right or left); (b) delay in contraction of both auricles with normal sinoauricular-auriculoventricular node conduction and resulting decrease in P-R interval; (c) conduction block to ventricles and left auricle; (d) auricular premature beats without ventricular contraction; (e) flutter of the right auricle with fibrillation of the left; (f) fibrillation, flutter, or flutter-fibrillation of both auricles.
3. Further possibilities of study of auricular disorders and the necessity of new interpretations are pointed out.
4. The experimental demonstration in animals of functional pathways through the auricular muscle needs further study in man in the clinic.

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## A MICROMETHOD FOR DETERMINATION OF TISSUE LIPIDS\*

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THE need for a convenient and accurate method for the determination of the lipid content of tissue has long been recognized. Several methods of lipid analysis have been developed. These methods give satisfactory results, but do not quite fill the requirements for routine analysis of a large number of samples, as they are time-consuming and laborious.

During the course of the past two years we have developed and used a method of lipid analysis which we find to be a marked improvement over the procedures in general use. This method saves time, requires only simple apparatus, and gives results that agree with those obtained by accepted procedures. Essentially it is the extraction method of Forbes and Irving<sup>1</sup> for the estimation of cholesterol in serum, applied to the extractions of the neutral fat and cholesterol of tissue, with the technique of Bloor and Snider<sup>2</sup> for the preparation of the extract for estimation of phospholipids. The estimation of neutral fat and cholesterol is made by the application of Bloor's<sup>3</sup> oxidative method to the chloroform-doucil extract. Total cholesterol is determined by the Leibermann-Burchard reaction, and the free cholesterol by the Sturges and Knudson<sup>4</sup> modification of the Schoenheimer-Sperry method.<sup>5</sup>

Phospholipids are calculated from the determination of the phosphorus in the alcohol-ether extract, or the phosphorus of the moist ether fraction, depending upon whether it is desirable to determine the total phospholipid or the lecithin-cephalin phospholipid. The sphingomyelin content of liver may be obtained from the difference between these two values (Kirk<sup>6</sup>).

### PREPARATION OF THE TISSUE EXTRACTS

Immediately after decapitating and draining the blood from the animal, the liver is removed and ground in a mortar. Thorough grinding is necessary, particularly when the livers are fatty, because the fat is frequently unevenly distributed.

### PREPARATION OF ALCOHOL-ETHER EXTRACT

One to 1.5 Gm. of finely ground liver is weighed on an analytical balance and transferred to a mortar containing 5 Gm. of fine sand. It is ground thoroughly and transferred with a spatula to a 125 c.c. Erlenmeyer flask. The mortar and pestle are washed twice with 20 c.c. of alcohol-ether (3:1) each time, the washing being added to the flask. The liver and sand mixture is thoroughly ground by stirring with a flat-tipped stirring rod. After an hour the flask is placed in a 70° C. water bath and allowed to boil for about two minutes. The alcohol-

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ether extract is filtered through a rapid filter paper into a 100 c.c. volumetric flask, and the residue is re-extracted twice with 25 c.c. of alcohol-ether as before. A final extraction is made with 20 c.c. of alcohol-ether, at room temperature. When filtering is complete, the alcohol-ether is added to the 100 c.c. mark. The extract is shaken thoroughly, and an aliquot is taken for phospholipid determination.

#### PREPARATION OF THE DOUCIL-CHLOROFORM EXTRACT

One to 1.5 Gm. of the finely ground liver is placed in a mortar containing 5 Gm. of doucil. The tissue and doucil are ground to a very fine powder and transferred to a 125 c.c. glass-stoppered pyrex bottle, and 100 c.c. of chloroform are added. The bottle is stoppered and shaken thoroughly. The shaking is repeated a number of times during the next few hours, and the extract is allowed to stand overnight. After thorough shaking the extract is filtered through a fat-free filter paper into another glass-stoppered bottle. The extract is now ready for use.

The advantage of this extract is that it contains only the neutral fat, cholesterol, and cholesterol esters of the tissue. A series of determinations has demonstrated that the phospholipids are completely removed by the doucil. This is of particular advantage in the colorimetric estimation of cholesterol by the Leibermann-Burchard reaction, as interference by phospholipids has been a criticism of this method of cholesterol estimation.

#### DETERMINATION OF LIPIDS

*Neutral Fat Plus Cholesterol.*—An aliquot of the chloroform extract, 1 c.c. to 5 c.c., depending upon the fat content of the liver, is measured into a 125 c.c. glass-stoppered pyrex flask. The flask is then placed in a water bath at 70° C., and all traces of solvent removed by a current of air. The neutral fat plus cholesterol residue is then determined by Bloor's<sup>3</sup> oxidative technique. Since the oxidation value per milligram of triglyceride in liver so closely approximates that of the fatty acids themselves, we have used Bloor's empirical relationship of 3.70 c.c. of 0.1 N sodium thiosulfate as equivalent to 1.0 mg. of lipid.

diluted to about 5 c.c. and decanted into the graduated tube. The flask is washed three times with about 2 c.c. of water, bringing the total volume in the tube to about 11 c.c. A standard tube containing 0.1 mg. of phosphorus is made up by taking 10 c.c. of the phosphorus standard and adding 1 c.c. of 60 per cent perchloric acid. If the phosphorus is low, it is advisable to make up another standard containing 0.05 mg. of phosphorus. To the standard and unknown 1 c.c. of 5 per cent ammonium molybdate is added, followed by 1 c.c. of 0.1 per cent aminonaphtholsulfonic acid solution. Water is then added to the 15 c.c. mark, and the solution is mixed thoroughly by inverting the tube several times. After standing for five minutes, the unknown is compared with the standard in a colorimeter. The total phospholipid is calculated by multiplying the phosphorus value by 25.

*Lecithin-Cephalin Phospholipid.*—Twenty cubic centimeters of the alcohol-ether extract are placed in a 50 c.c. Erlenmeyer flask and evaporated to dryness on a 70° C. water bath with a current of air. After removing from the water bath, 0.3 c.c. of water is added, followed by a few drops of moist ether. The flask is thoroughly shaken and the ether is evaporated off. Ten cubic centimeters of moist ether are added. The flask is shaken and placed in a water bath until the ether boils and the volume is reduced to 5 c.c. The moist ether solution is decanted into a 25 c.c. glass-stoppered graduated cylinder, care being taken that none of the aqueous layer is transferred. The extraction is repeated twice, thus making a total volume in the cylinder of between 15 and 20 c.c. Moist ether is added to the 20 c.c. mark. The flask is stoppered, shaken thoroughly, and allowed to stand overnight. A slight cloudy precipitate settles out. Without disturbing the precipitate, a 5 c.c. aliquot is removed, and the phosphorus content is determined as described for total phospholipid.

The sphingomyelin content of the tissue can be determined by the difference between the total and the lecithin-cephalin phospholipid.

*Total Cholesterol.*—To 5 c.c. of the chloroform extract in a 10 ml. glass-stoppered cylinder are added 2 c.c. of a 20:1 mixture of acetic anhydride with concentrated sulfuric acid. The sulfuric acid should be added to the acetic anhydride, shaken thoroughly, and cooled to 25° C. immediately before use. It has been found that much better results are given by this method than by the usual procedure of adding 0.1 c.c. of sulfuric acid to the chloroform acetic anhydride mixture. After adding the acetic anhydride-sulfuric acid mixture, the cylinders are stoppered, shaken thoroughly, placed in a beaker of water at 25° C., and put in the dark for ten to fifteen minutes for color development. They are then compared in a Duboseq colorimeter with a standard containing 0.2 mg. of cholesterol prepared at the same time and under the same conditions. A Wratten filter No. 71A and a permanent standard, as recommended by Shapiro, Lerner, and Posen,<sup>8</sup> are used for the comparison of color.

*Free Cholesterol.*—A 5 c.c. aliquot of the chloroform extract is placed in a 15 c.c. centrifuge tube and the chloroform removed by placing it in a 70° C. water bath and directing a current of air on the surface of the liquid. Bumping may be prevented by placing a small stirring rod in the tube. When the chloroform has been completely removed, 1 c.c. of alcohol-acetone (1:1) solution

is added. The lipid is dissolved by stirring. Three drops of 5 per cent hydrochloric acid and 1 c.c. of 0.3 per cent digitonin in 90 per cent alcohol are added. The stirring rod is removed and placed so that it can be returned to its tube; the tube is centrifuged fifteen minutes at 2,000 r.p.m. The supernatant liquid is decanted and the precipitate and stirring rod are washed with acetone-ether (1:2), the centrifuging is repeated, and the supernatant liquid is removed by suction. The washing is repeated twice with anhydrous ether.

The last trace of ether is removed with a current of dry air on a water bath, and the cholesterol digitonide is dissolved in 1 c.c. of acetic acid by placing the tube in a 60° C. water bath. After removing from the bath and cooling, 2 c.c. of acetic anhydride-sulfuric acid (20:1) mixture, as described for total cholesterol, is added, and the tube is placed in a 25° C. bath and kept in the dark for twenty-five minutes. It is compared with a standard containing 0.2 mg. of cholesterol, prepared by treating a standard cholesterol solution in chloroform exactly as above. The standard cholesterol and the unknown are usually set at 10 mm., and the readings are taken of the permanent standard.

Typical results obtained by the methods here described are compared with those obtained with well-established methods in Tables I, II, and III.

#### DISCUSSION

That Bloor's oxidative method of determining the total fatty acids and cholesterol of tissue can be applied to the chloroform extract is indicated by the close agreement shown in Table I between the values obtained by Bloor's method on the alcohol-ether extract and those obtained by calculating these constituents from the values obtained from the fatty acids of the total phospholipid, and the neutral fat plus cholesterol of the chloroform extract.

TABLE I  
COMPARATIVE DETERMINATIONS OF TOTAL FATTY ACIDS PLUS CHOLESTEROL IN NORMAL AND FATTY LIVERS OF RATS

The values are expressed as per cent of fresh tissue

TOTAL PHOSPHOLIPIDS	NEUTRAL FAT PLUS CHOLESTEROL	TOTAL FATTY ACIDS PLUS CHOLESTEROL	
		AUTHORS' METHOD	BLOOR'S METHOD
3.30	23.8	25.1	25.1
3.04	28.0	28.8	29.2
2.79	21.8	22.7	23.4
2.65	32.2	32.6	33.2
3.92	4.5	7.0	6.4
4.10	5.8	8.4	8.4
3.02	14.5	16.0	16.6
3.66	7.6	9.8	9.7
2.42	29.0	29.4	31.2
2.20	35.0	35.0	35.4
3.60	8.3	10.4	9.9
3.12	13.6	15.2	15.2
2.70	29.3	29.9	28.6
3.58	0.89	3.3	3.3
3.41	0.92	3.2	3.1
3.54	0.90	3.3	3.5
3.22	0.74	2.9	3.0
3.56	0.89	3.4	3.4
2.79	1.10	3.0	3.0
3.54	0.72	3.1	3.0

TABLE II

COMPARATIVE DETERMINATIONS OF FREE AND TOTAL CHOLESTEROL IN NORMAL AND FATTY LIVERS OF RATS

The values are expressed as per cent of fresh tissue

TOTAL CHOLESTEROL			FREE CHOLESTEROL	
DOUCIL-CHCl <sub>3</sub> EXTRACT		ALCOHOL-ETHER EXTRACT	DOUCIL-CHCl <sub>3</sub> EXTRACT	ALCOHOL-ETHER EXTRACT
AUTHORS' METHOD	DIGITONIDE METHOD	DIGITONIDE METHOD	DIGITONIDE PRECIPITATION	DIGITONIDE PRECIPITATION
0.19	0.19	0.19	0.17	0.17
0.20	0.21	0.23	0.19	0.19
0.24	0.23	0.24	0.17	0.18
0.19	0.19	0.20	0.16	0.15
0.22	0.22	0.22	0.20	0.20
0.25	0.27	0.22	0.21	0.20
0.21	0.21	0.20	0.16	0.16
1.15	1.15	1.14	0.22	0.22
0.41	0.44	0.46	0.22	0.24
0.90	0.90	0.94	0.24	0.25
0.89	0.87	0.88	0.17	0.19
0.67	0.65	0.69	0.17	0.19
0.36	0.34	0.35	0.18	0.19
0.77	0.76	0.80	0.16	0.18
0.87	0.86	0.90	0.21	0.23
0.38	0.38	0.40	0.22	0.23
0.87	0.87	0.81	0.21	0.20

Note.—The digitonide method used is a slight modification of Schoenheimer and Sperry's method.

TABLE III

TYPICAL RESULTS OF COMPLETE LIPID ANALYSIS OF RAT LIVERS

The values are expressed as per cent of fresh tissue

TOTAL PHOSPHO-LIPID	LECITHIN-CEPHALIN	SPHINGO-MYELIN	NEUTRAL FAT AND CHOLESTEROL	TOTAL CHOLESTEROL	FREE CHOLESTEROL	ESTER CHOLESTEROL	IODINE NUMBER OF NEUTRAL FAT AND CHOLESTEROL
2.31	1.92	0.39	33.3	0.54	0.15	0.39	88
3.34	2.67	0.67	12.1	0.51	0.18	0.33	94
3.35	2.69	0.66	0.9	0.20	0.18	0.02	117

Repeated extractions with moist ether of the residues from evaporation of the chloroform extracts of a large number of livers has demonstrated that the chloroform extract contains no trace of phospholipids. Only neutral fat, cholesterol, and cholesterol esters are present in this extract.

Completeness of extraction is demonstrated by the agreement between the total fatty acid values on the two extracts, as given in Table I, and also by the agreement of the cholesterol values in Table II, as determined by the digitonide method on the chloroform extract and the alcohol-ether extract.

The determination of phospholipid by calculation from the phospholipid phosphorus is in general use, and according to Kirk<sup>6</sup> can be used for determining total phospholipid (alcohol-ether soluble phospholipid), lecithin and cephalin (moist ether-soluble phospholipids), and sphingomyelin (phospholipid soluble in alcohol-ether and insoluble in moist ether).

There are a number of methods in use for the determination of cholesterol. Criticisms of the methods of cholesterol determination are so common that there

seems to be no necessity of detailed discussion of the merits and demerits of the various procedures. The Schoenheimer-Sperry method, which appears to be the method in common use at present, was chosen as a standard for evaluating our procedure for determination of free and total cholesterol. As shown in Table II, the method described for estimation of free and total cholesterol gave values that agree with those obtained by the Schoenheimer-Sperry method.

Some difficulty was experienced in the precipitation of the cholesterol digtonide, in the determination of total cholesterol, when applying the Schoenheimer-Sperry method to fatty livers. Frequently the total cholesterol of fatty livers, produced either by carbon tetrachloride poisoning or by a high fat diet, gave values lower than those obtained for free cholesterol. Results could not be duplicated, and commonly ranged from 10 per cent to 60 per cent of the value obtained for total cholesterol by the Leibermann-Burchard reaction applied to the chloroform extract. Sturges and Knudson<sup>4</sup> reported similar experiences and suggested that this could be overcome by adding 3 drops of 5 per cent hydrochloric acid in excess after neutralizing the solution following saponification. They used only normal tissues in their experiments. We found that this procedure would give good results on normal livers, but made no improvement when the method was applied to fatty livers. The substitution of 1 drop of 50 per cent sodium hydroxide for the potassium hydroxide in the saponification solved this difficulty, and gave satisfactory duplicate results at all times. Furthermore, these results have always agreed with those obtained using the method described in this paper.

The chloroform extract has been found to be suitable for determination of iodine numbers by the method of Yasuda.<sup>9</sup>

#### SUMMARY

1. The preparation of a chloroform extract of liver suitable for the determination of neutral fat, total cholesterol, free cholesterol, ester cholesterol, and iodine number of the neutral fat plus cholesterol, is described.

2. A convenient method for the determination of total phospholipids, lecithin-cephalin phospholipids, and sphingomyelin content of liver is presented.

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## FATAL REACTIONS TO ADMINISTRATION OF SULFONAMIDE DRUGS\*

### REPORT OF FIVE CASES

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IN THE wake of numerous successful and life-saving results from the use of the sulfonamide drugs, there continue to appear reports and mention of occasional fatal reactions. So far these reports do not seriously challenge the use of these compounds, but serve to warn against the use of such potent agents without adequate indication or supervision. The reported fatal complications have been granulocytopenia, hemolytic anemia, and liver damage. Since the introduction of these drugs, we have encountered examples of each of these complications in our autopsy service at the Los Angeles County Hospital. To increase slightly the fund of available information concerning fatal reactions, five deaths with autopsy are reported. Three of the patients died from granulocytopenia, one from apparent liver and kidney damage, and one from hemolytic anemia. In four patients sulfanilamide was the drug used. In the fifth patient, who died of granulocytopenia, sulfapyridine was used.

#### FATAL GRANULOCYTOPENIA INDUCED BY SULFANILAMIDE

The most recent review of fatal granulocytopenia associated with sulfanilamide therapy<sup>1</sup> brought the total number of reported cases to ten. At about the same time, two additional cases were recorded by Ottenberg.<sup>2</sup> Other cases have been reported by Corr and Root,<sup>3</sup> Sailer,<sup>4</sup> and Garvin.<sup>5</sup> The following are summaries of the clinical records and necropsy findings in two additional cases.

CASE 1.—(Los Angeles County Hospital, No. 635-179.) A 15-year-old school boy entered the hospital on November 2, 1938, with a scarlatinal rash and fever of one day's duration. Temperature was 100° F. and enanthem was noted in the pharynx. Urinalysis was negative and blood count revealed hemoglobin of 85 per cent (Salili), erythrocytes 4.1 million, and leucocytes 12,500 per c. mm., with 85 per cent polymorphonuclear cells and 15 per cent lymphocytes. Sulfanilamide, 6.4 Gm. daily, was given for seven days until the temperature returned to normal. After two days without treatment a scarlatinal eruption recurred and sulfanilamide was again started and administered for the rest of the patient's stay in the hospital. The total amount given was 100.6 Gm. over an eighteen-day period. The day before dis-

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charge the patient's blood count showed 2.6 million erythrocytes and 5,030 leucocytes per c. mm., with 74 per cent polymorphonuclear cells, 24 per cent lymphocytes, and 2 per cent monocytes. The blood sulfanilamide level was 5.5 mg. per 100 c.c. on this day.

After discharge the patient felt well and took no other drugs, but within three days he was again acutely ill, with sore throat and temperature of 104° F. He re-entered the hospital on November 24, 1938. Mild hyperemia of the pharynx and reddened left eardrum were the only physical findings noted. His blood count, however, now showed hemoglobin of 60 per cent (Sahli), erythrocytes 4.0 million per c. mm., and leucocytes only 675 per c. mm., with no polymorphonuclears or monocytes. Blood culture, taken on entry, yielded no growth. Blood transfusion, leucocytic extract, and yellow bone marrow were administered with no improvement. The following day his hemoglobin was 58 per cent (Sahli), erythrocytes were 3.6 million per c. mm., and leucocytes 550 per c. mm. He died with no further change in his condition. The clinical diagnosis was granulocytopenia, probably induced by sulfanilamide.

At necropsy there was only slight gross evidence of pharyngitis. The paranasal sinuses, aural cavities, meninges, and brain were normal. A few small areas of partial consolidation were present in each lung. The liver weighed 2,050 Gm. and its cut surface had obscured markings and a peculiar salmon pink color frequently seen in the livers of patients who had received sulfanilamide shortly before death. Chemical analysis of a portion of the liver revealed 2.5 mg. per cent of free sulfanilamide (Table I). The spleen weighed 250 Gm. and its cut surface was soft, blue black, with obscured Malpighian markings. The bone marrow of the sternum, vertebral bodies, and the upper end of the femur was hyperemic.

Microscopic examination of the consolidated areas of the lungs showed hyperemia, intra-alveolar edema, occasional mononuclear cells, but no polymorphonuclear leucocytes. The liver showed moderate parenchymatous degeneration and slight fatty change. The glomeruli of the kidneys appeared unchanged. Many of the convoluted tubules showed severe cloudy swelling with some karyolysis. The splenic pulp was markedly hyperemic with striking diminution of granular leucocytes. Increased iron-containing pigment was demonstrated by the Prussian blue stain. Sections of the vertebral marrow and smears of the sternal marrow showed reduction in the granular series with maturation arrest at the myelocyte level. Erythroblasts were abundant and numerous plasma cells were also noted.

CASE 2.—(Los Angeles County Hospital, No. 398-057.) A Caucasian housewife, aged 33 years, after several recurrent attacks of sore throat early in September, 1937, was seen by a private physician, who made a diagnosis of "streptococcal sore throat" and started treatment with sulfanilamide. The patient failed to return to her physician for observation, but continued to take sulfanilamide to a total of 64 Gm. over a period of three weeks. She then entered the hospital because of failure to improve. She was acutely ill, with a temperature of 104.3° F., pulse 140, and respirations 24 per minute. The pharynx was obscured by a gray membrane from which no bacteria were obtained on smear. Laboratory findings included normal urine and blood urea nitrogen of 12 mg. per 100 c.c. The blood count revealed hemoglobin 7.5 Gm., erythrocytes 2.4 million, platelets 266,000, leucocytes 550 per c. mm., with 97 per cent lymphocytes, 3 per cent monocytes, and no polymorphonuclear neutrophils. Treatment consisted of blood transfusions, pentonucleotide, and liver extract. The patient's temperature remained high until death on the third day after entry to the hospital.

At autopsy moderate jaundice and cervical lymphadenopathy were externally evident. The pharynx and tonsils were partially necrotic and covered with a yellow-gray membrane. In both lungs there were considerable hyperemia and edema. The liver weighed 2,200 Gm. Surfaces made by sectioning were softer than normal and had obscured markings. The spleen weighed 300 Gm. The pulp was red and softened, obscuring the normal markings. The kidneys together weighed 300 Gm. and showed cloudy swelling. The bone marrow of the sternum and bodies of the lumbar vertebrae and femur appeared grossly normal. Histologically, the necrotizing pharyngitis was characterized by an absence of polymorphonuclear leucocytes. In the liver there was parenchymatous degeneration associated with necrosis of scattered cells in the centers of some lobules. The spleen showed an increase of mononuclear elements. The bone marrow, unfortunately, was not examined microscopically.

## COMMENT

It appears likely that the fatal granulocytopenia in these two patients is attributable to sulfanilamide. They resemble previously reported cases. The onset of granulocytopenia was noted about three weeks after treatment was instituted. In the second patient the dosage used was average. In the first patient the daily dosage (6.4 Gm.) was higher than that generally recommended by Long.<sup>6</sup> The total dosage used, 101 Gm., was twice the average in the reported cases of granulocytopenia, but it apparently was excreted adequately, since the blood level near the conclusion of treatment was only 5.5 mg. per cent. The continuance of sulfanilamide for a week or more after the subsidence of the acute symptoms, as was done in this patient, is considered good practice in the avoidance of complications following scarlet fever. In this connection, it is of interest to note that the leucocyte count of the blood at the cessation of treatment was only slightly below normal. It would appear, as with amidopyrine, that fatal granulocytopenia may evolve after discontinuing the use of the offending drug. With the second patient it is, of course, possible that agranulocytic angina was already present when sulfanilamide was started, since no early blood counts were available. The patient did not return to her physician, but the total dosage taken before entry to the hospital was not excessive. Another point, emphasized by others as a poor prognostic sign, is the absence of monocytosis during the period of granulocytopenia. This was also noted in both cases reported here. The iron pigmentation of the spleen has been noted both in reported cases and in experimental observations.<sup>7</sup> The bone marrow picture is similar to that already described in sulfanilamide granulocytopenia.<sup>1</sup>

## FATAL GRANULOCYTOPENIA INDUCED BY SULFAPYRIDINE

The introduction of the new compound, sulfapyridine, was accompanied by the warning that toxic effects similar to those produced by sulfanilamide might be anticipated,<sup>8</sup> and case reports of granulocytopenia began to appear soon thereafter.<sup>9-11</sup> In at least four instances the granulocytopenia was fatal. Rosenthal and Vogel's<sup>12</sup> patients were children who received 95 and 35 Gm. of the drug for *Staphylococcus aureus* sepsis and lobar pneumonia, respectively, and developed granulocytopenia in about two weeks. Autopsy findings are not recorded. Dolgopol and Hobart<sup>13</sup> have reported a similar case in which fatal granulocytopenia complicated the treatment of type VII pneumococcal pneumonia with 49 Gm. of sulfapyridine. At autopsy severe depression of bone marrow was found. Johnston's<sup>9</sup> patient had been treated for acute *Streptococcus viridans* bacterial endocarditis complicating puerperal sepsis, with 54 Gm. of sulfapyridine over an eleven-day period. Neutropenia soon appeared and at necropsy, several days later, the endocarditis was still evident. The following is a report of a somewhat similar case.

CASE 3.—(Los Angeles County Hospital, No. 648-416.) C. B., a white male of 53, entered the hospital on February 16, 1939, with fever, weakness, and fleeting joint pains of five weeks' duration. Following a peritonsillar abscess one year previously, he had progressive weakness, cough, and weight loss. There was no past history of rheumatic fever. Physical findings included low-grade fever, regular pulse, rough systolic apical murmur, and palpably enlarged spleen. Laboratory examination revealed normal urine, blood count of 4.3

million erythrocytes per c. mm., 75 per cent hemoglobin (Sahli), and 7,500 leucocytes with 95 per cent polymorphonuclear cells. Several blood cultures were positive for *Streptococcus viridans*. The clinical diagnosis was subacute *Streptococcus viridans* endocarditis.

The patient was given 88.5 Gm. of sulfapyridine between February 22 and March 15, 1939. A temporary remission of fever was noted during the first few days of treatment. On March 2 the blood sulfapyridine was 8.2 mg. per 100 c.c., and the blood culture was positive for *Streptococcus viridans*. On this day blood count revealed hemoglobin of 60 per cent (Sahli), leucocytes 11,250 per c. mm., with 80 per cent polymorphonuclear cells and 30 per cent lymphocytes. No further blood count was done until March 15, when the patient complained of sore throat. Blood count now showed a hemoglobin of 50 per cent (Sahli), erythrocytes 2.7 million per c. mm., and leucocytes 1,100 per c. mm., with 78 per cent lymphocytes and 22 per cent polymorphonuclears. Sulfapyridine was immediately discontinued and blood transfusion was given. The anemia and leucopenia persisted, the last leucocyte count being 1,450 per c. mm. on the day of death (March 21, 1939).

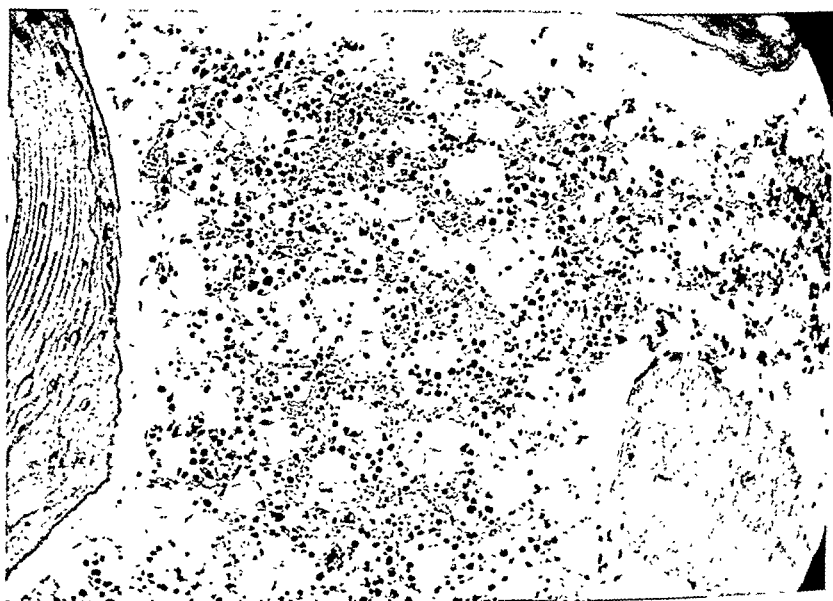


Fig. 1.—Vertebral bone marrow. Giemsa stain ( $\times 235$ ). Fibrinoid necrosis and reduction of myeloid series is shown.

At necropsy numerous petechiae and moderate jaundice were noted in the skin and mucous membranes. No areas of softening were noted in the brain. The heart weighed 330 Gm. All chambers were somewhat dilated, the valve measurements were within normal limits, but the mitral leaflets were slightly thickened and shortened, and the tips of the papillary muscles were fibrosed. In addition, there were several small soft light brown vegetations on the anterior leaflet of the mitral valve. These were situated near the attachment of several of the chordae tendineae onto which they extended. None was over several millimeters in diameter. The liver appeared grossly normal. The spleen weighed 370 Gm. and showed, in addition to softened pulp, a recent infarct 2 cm. in diameter near one surface. The kidneys together weighed 370 Gm. and showed a few small infarcts. Apart from moderate hyperemia of the lungs and some small mucosal ulcerations in the esophagus, the other organs showed no striking alteration. The bone marrow of the sternum, vertebral bodies, and long bones was grossly normal.

Histologic examination showed several changes of interest. Section made through the anterior mitral leaflet in the region of the small gross vegetation showed moderate thickening of the valvular endocardium, with scattered fibrinous deposits on the surface. In some of these areas bacterial masses were seen. In the subendocardial tissue there were occasional

collections of mononuclear cells about small thickened blood vessels. In the section taken through the area of gross infarction in the spleen there were numerous clusters of bacteria, generally in blood vessels within the infarcted area. No wall of leucocytes occurred at the margin of the infarct, and polymorphonuclears were generally scarce throughout the uninvolved portions. Large quantities of iron pigment were also demonstrated throughout the entire spleen. In the kidney section the convoluted tubules showed moderate cloudy swelling and fatty change. In the esophageal ulcerations, necrosis, bacterial masses, and lymphocytic exudate were noted.

Microscopic examination of the vertebral marrow showed a reduction in cellular elements, particularly in the myeloid series. There appeared to be a maturation arrest at the myelocyte level. Erythroblasts were present in normal numbers. In addition, it was of interest to note areas in which poorly stained marrow cells were mixed with fibrin, producing the appearance of focal necrosis in the bone marrow (see Figs. 1 and 2).

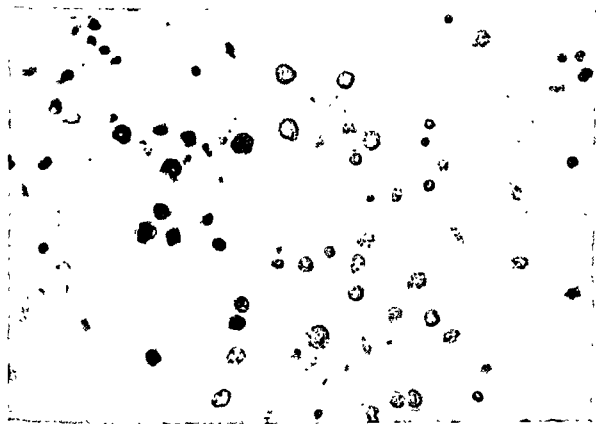


Fig. 2.—Vertebral bone marrow. Giemsa stain ( $\times 960$ ) Erythroblasts, lymphocytes, reticulum cells, and occasional myelocytes are shown.

#### COMMENT

Although Case 3 was probably doomed by a fatal disease, the immediate cause of death was, no doubt, granulocytopenia, probably induced by sulfapyridine. More frequent blood counts during the period of treatment might have avoided this complication. The clinical aspects and autopsy findings with regard to the granulocytopenia were generally similar to those described in the patients treated with sulfanilamide. Sulfapyridine seems to have the same potential danger in the production of granulocytopenia as does sulfanilamide. Rather large doses over a period of two or three weeks may result in fatal granulocytopenia. Two side lights with regard to the endocarditis are of interest. One is the early improvement in the febrile state after the drug was started, although blood cultures remained positive. The other is the unusually small size of the vegetations. We have recently seen at autopsy in another patient who died of cardiac failure with subacute bacterial endocarditis, apparent reduction of vegetations to almost microscopic size after treatment with sulfapyridine.

## FATAL HEMOLYTIC ANEMIA INDUCED BY SULFANILAMIDE

Hemolytic anemia is recognized as a relatively frequent complication of sulfanilamide therapy that develops after a few days of drug administration, and responds readily to treatment, particularly withdrawal of the drug and blood transfusion. Two fatal cases of hemolytic anemia are reported in the literature.<sup>14, 15</sup> The following is a report of another such case.

CASE 4.—(Los Angeles County Hospital, No. 247-892.) A 51-year-old white male, carpenter, was admitted to the Hospital on June 12, 1939, with a typical but not severe erysipelas, involving the external nares, of three days' duration. The past history was negative except for anginal attacks for three and one-half years. His temperature was 99.2° F., and he did not appear acutely ill. Urinalysis showed no albumin or sugar. Blood Wassermann was negative. His blood count on admission was hemoglobin 144 per cent (Sahli), erythrocytes 4.42 million, and leucocytes 14,900. Icterus index on June 13 was less than 10 units. During the next three days the patient received 25 Gm. of sulfanilamide, following which the blood level reached 7 mg. The following day deep jaundice was noted and the blood count now showed hemoglobin 42 per cent (Sahli), erythrocytes 1.75 million, and leucocytes 33,750, with 82 per cent polymorphonuclear leucocytes. The icterus index was 80 units. On this day the urine was noted to be very dark brown, with a positive benzidine test and no red blood cells. The urinary reaction was not recorded.

The clinical diagnosis was erysipelas, complicated by acute hemolytic anemia induced by sulfanilamide. Treatment consisted of repeated small transfusions of both whole and citrated blood. The patient belonged to group O, and received a total of eleven transfusions between June 16 and June 22. A severe chill followed the first transfusion, but no reaction occurred after any of the others. During the first few days his general condition was poor, with dark urine, prominent icterus, and considerable lethargy. On June 20 the urine was clear, amber, and showed 150 to 200 red blood cells per high dry field in the centrifuged sediment. During the next week the patient's anemia improved and his jaundice diminished. There was a progressive increase in erythrocytes to 4.32 million on June 29, with 72 per cent hemoglobin. The leucocytes fluctuated considerably between outer limits of 33,000 and 9,800 per c. mm.

It thus appeared that the patient had recovered from his hemolytic anemia, but during the next week his condition grew worse, with two major manifestations. One was the occurrence of subcutaneous abscesses on the scalp and hands; the other was the development of progressive stupor following an epileptiform convulsion on June 28. His temperature, which had returned to normal during the first week, remained so, except for one rise to 101° F. His stupor deepened into coma and he died on July 11, 1939.

Autopsy was performed seventeen hours after death by Dr. Clara Margoles. Several subcutaneous abscesses, some draining thick pus (staphylococci in Gram stain smear) were still present. Small abscesses had also developed at the site of previous erysipelas of the nose. No icterus was noted. No lesions were found in the brain. Severe coronary sclerosis was present. The lungs showed patchy bronchopneumonia. The liver weighed 1,200 Gm.; cut surfaces were firm and dark brown. The spleen weighed 110 Gm.; cut surfaces were flabby but not soft, with pulp not unusually prominent. The kidneys together weighed 320 Gm.; capsules stripped readily from an unusually brick red-brown surface, which on cut surface showed similar pigmentation, particularly in the medullary portions. In addition, the parenchyma was softened, and a number of milary abscesses were noted. The bone marrow of the vertebrae was brown, and that of the femur was fatty. The post-mortem blood creatinine was 5.0 mg. per 100 c.c.

The microscopic examination of the liver showed moderate cloudy swelling, slight fatty change, and with the Turnbull stain, large quantities of iron-containing pigment, often located in cells lining the sinusoids. In the spleen there was also a marked increase over normal in the amount of iron-containing pigment present, both intra- and extracellularly. The pulp was crowded with leucocytes, and there was some evidence of erythrophagocytosis.



Fig. 3.—Kidney cortex ( $\times 100$ ). Tubular degeneration and obstruction of lumen in cortex and medullary rays by hemoglobin casts



Fig. 4.—Kidney medulla ( $\times 100$ ). Tubular degeneration and obstruction of lumen in cortex and medullary rays by hemoglobin casts.

The most striking changes were in the kidney. Here, in addition and apart from the miliary abscesses occasionally seen, there was marked tubular degeneration of the convoluted tubules and the loops of Henle. In the lumina of these tubules was disintegrated epithelium, as well as masses interpreted as blood pigment (see Figs. 3 and 4). Amorphous material was also noted in some of the collecting groups. A small amount of this material took the iron stain. Some of the epithelial casts showed partial calcification. There was no evidence of regeneration in the scattered tubules, which showed considerable epithelial necrosis. Glomeruli and blood vessels appeared essentially normal. Section of the vertebral marrow stained with hematoxylin and eosin showed a normal cellular distribution, except, perhaps, for some increase in nucleated blood cells.

#### COMMENT

The case herein reported conforms to the previous descriptions of the hemolytic anemia reaction. The anemia develops rapidly, within a few days after the administration of ordinary doses of the drug. In the two previously reported fatal cases death was attributed largely to the anemia, and nonspecific changes secondary to hemolysis and anemia were described at autopsy. In the present case, it is noted that the patient had recovered from the anemia, but died from uremia. From the changes in the kidney, there is reason to believe that the uremia occurred by obstruction and also direct damage to the kidney tubules by products of hemoglobin disintegration, e.g., acid hematin. Hemolytic anemia thus in this case caused death by delayed effects on the kidney. This effect is not specific for sulfanilamide, and has been described for other conditions, particularly after incompatible blood transfusions and in experimental hemoglobinemia. According to DeGowin, Warner, and Randall,<sup>16</sup> the renal damage is due not only to obstruction, but partly to direct injury to the tubules. In this connection, it is interesting to note that Wood's<sup>14</sup> patient was irrational, and vomited and bled from the gums before death; Koletsky's<sup>15</sup> patient died in coma. Some pigment deposit was noted in the kidney in the latter case.

Several important points need emphasis in regard to the treatment of the acute hemolytic anemia reaction. Blood transfusion is generally considered as indicated if the anemia becomes severe, as it usually does. It has been noted by Sharpe and Davis<sup>17</sup> that hemolytic reactions may complicate blood transfusion for hemolytic anemia, perhaps more than by mere coincidence. In the patient here reported a severe chill followed the first transfusion used. Additional hemolysis of the donors' corpuscles may have occurred at this time and added to the injury of the kidney produced by sulfanilamide hemolysis of the patient's corpuscles. A similar experience occurred in a case of hemolytic anemia and uremia recently reported,<sup>18</sup> where it was decided that the blood transfusion, rather than the sulfanilamide, was responsible. Attention has recently been called by Antopol and others<sup>19</sup> to another interesting phenomenon developing in the blood following sulfanilamide therapy, namely, the appearance of auto-agglutination together with hemolytic anemia. This property appeared when the blood was cooled and might be precipitated by giving blood transfusion of unwarmed citrated blood. Theoretically, there is potential danger in such a phenomenon to the kidney, by the plugging of glomeruli, as has been described in other instances of autohemagglutination, e.g., multiple myeloma.

The only way to avoid renal damage as a consequence of hemolytic anemia, is to alkalinize the urine in order to prevent the deposition of the obstructing



pigments in the renal tubules. It would appear that in severely ill patients likely to require both sulfanilamide and blood transfusions, or even in patients already showing signs of hemolytic anemia, rapid alkalization of the urine should be carried out.

#### FATAL LIVER AND KIDNEY DAMAGE INDUCED BY SULFANILAMIDE

The probable causal relationship of the sulfonamide drugs to fatal granulocytopenia appears reasonably established by the case reports, and is in keeping with the chemical nature of these drugs, and with observations on their effect on the leucocyte count. Hemolytic anemia is also well established as a more frequent, but less dangerous, complication. Severe liver damage is a more recently described, and not as well established, toxic manifestation. Long, Bliss, and Feinstone<sup>6</sup> indicate that jaundice (without anemia) occurred in 0.6 per cent of 307 adult patients treated, and was controlled by stopping the use of sulfanilamide. Hageman and Blake<sup>20</sup> noted one case of hepatitis, possibly due to sulfanilamide in 114 treated patients. More frequently, jaundice has occurred incidental to the well-established hemolytic anemia reaction. However, there have been a number of recent reports indicating an apparent primary liver damage following the use of sulfanilamide<sup>21-27</sup>. The case reported by Fitzgibbon and Silver<sup>22</sup> was quite interesting. After receiving 44 Gm. of sulfanilamide for gonococcal urethritis, their patient had a generalized skin eruption, and was perhaps slightly jaundiced. Six weeks later, after taking only 1 Gm. of sulfanilamide for a recurrent urethral discharge, he developed dermatitis, this time associated with enlarged liver and severe jaundice followed by oliguria. The highest icterus index before recovery was 200, at which time the blood nonprotein nitrogen was 75 mg. per cent. Garvin's report<sup>23</sup> presented five cases in which the sulfanilamide appears to have been responsible for liver damage. The dosages of sulfanilamide employed were not excessive (maximum 50 Gm.) and were used in a conventional manner for diseases in themselves not serious. Jaundice was the prominent feature (highest icterus index noted was 160) and could not be explained on the basis of hemolytic anemia. It is interesting to again note the frequent association of dermatitis with the hepatitis. One of the patients died, but necropsy was not obtained. He also referred to two deaths with jaundice, commented on without detailed description in the report of Bannick and co-workers.<sup>25</sup> The week following Garvin's report, a case of acute yellow atrophy following sulfanilamide medication was reported by Cline<sup>26</sup> in the same journal. The patient was a white male of 18 years, who took an unknown quantity of sulfanilamide for gonorrheal urethritis, developed a clinical picture of hepatic insufficiency with an icterus index of 166, nonprotein nitrogen 33, and showed severe liver and kidney damage at necropsy. Greene and Hotz,<sup>27</sup> in their recent review of literature on the liver and biliary tract, mention four additional cases, with two deaths and one necropsy. Case 5 is a summary of the important clinical and necropsy data in an additional case of apparent liver and kidney damage following the use of sulfanilamide.

CASE 5.—(Los Angeles County Hospital, No. 607-856.) The patient was a Filipino male, aged 23 years, who entered the hospital because of pain, swelling, and dysfunction of the

left shoulder, knee, and hip joints. The referring physician stated that the patient was a periodic heavy drinker, but not in recent weeks. There was no known history of venereal disease or treatment, and no previous drug therapy for the present illness. Physical examination revealed a low-grade fever, evidence of acute polyarthritis, and purulent discharge from the urethra. Blood count showed hemoglobin 95 per cent (Sahli); leucocytes 11,000, with 80 per cent polymorphonuclear leucocytes. Gram-negative intracellular diplococci were found in the urethral discharge. The blood Wassermann and Kahn tests were positive. A small amount of serous fluid was aspirated from the left ankle joint, which contained a few leucocytes, but no bacteria on smear or culture.

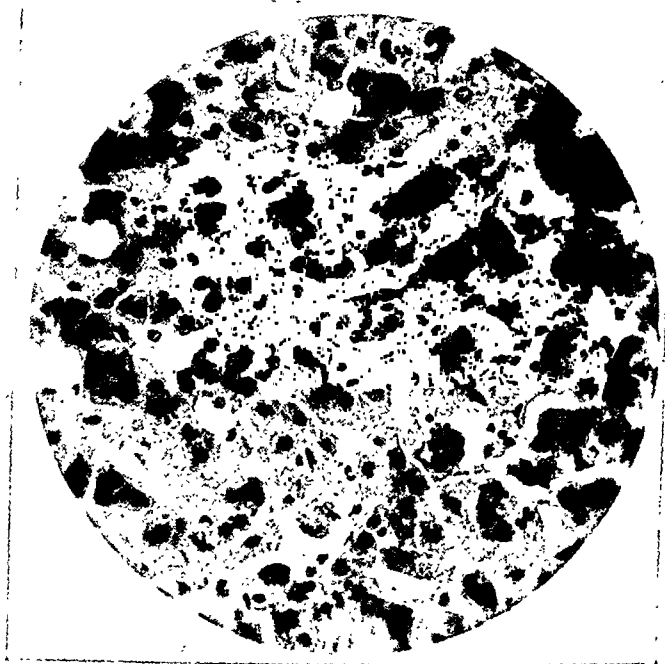


Fig. 5.—Liver, showing fragmented and swollen cell cords with cellular degeneration ( $\times 400$ ).

The clinical diagnosis was gonococcal urethritis, with early polyarthritis, and the patient was started on sulfanilamide. Between May 6 and May 11, 1938, thirty-eight doses, totaling 34 Gm., were given. No antisiphilic treatment was administered. On May 10 (one day before sulfanilamide was discontinued) jaundice was noted for the first time, but had probably previously been present and masked by the patient's native color, since blood drawn on that day had an icterus index of 220, and gave a prompt positive direct van den Bergh reaction with 29.1 mg. per cent of bilirubin. The urine contained albumin, bile, and a few hyaline casts. The patient's jaundice deepened, a maculopapular rash developed on May 14 (thought to look more like drug rash than secondary syphilis), and the patient became irrational. A blood transfusion was given without apparent improvement. The urethritis had meanwhile subsided. He developed Cheyne-Stokes respiration and died in coma.

At autopsy, jaundice and slight swelling of the left shoulder joint were still evident. No gross lesions were noted in the brain, heart, aorta, or gastrointestinal tract. The lungs were moderately hyperemic and edematous, but without gross consolidation. The liver weighed 2,300 Gm. Its external surface was smooth with tense capsule. Surfaces made by sectioning were softer than normal, with somewhat obscured lobular markings of mottled green to light brown. The gall bladder was of average size and thickness, but contained only a small quantity of inspissated bile. The biliary tract and portal system were normal. The spleen weighed 230 Gm., its pulp was prominent, dark purple, and somewhat softened. The kidneys together weighed 575 Gm. and were similar in appearance, each being about twice

normal size. The tense capsule stripped readily from a swollen, bile-stained surface. Surfaces made by sectioning were swollen, soft, with markings accentuated by bile staining. The urinary bladder contained a few drops of bile-stained urine. Prostate and seminal vesicles appeared grossly normal, as did the urethra throughout its extent. Small quantities of slightly bile-stained fluid were present in the right ankle and left shoulder joints, the synovia of the latter being slightly thickened. The post-mortem blood nonprotein nitrogen was 142, creatinine 14.4, and urea nitrogen 85 mg. per cent. No sulfanilamide was detected in the post-mortem blood; 2 mg. per cent were found in the liver (Table I).

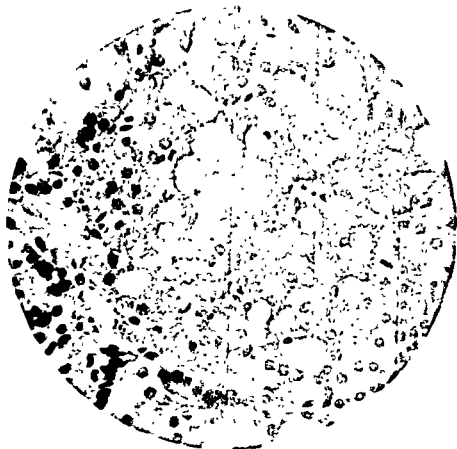


Fig. 6.—Kidney, showing focal necrosis of convoluted tubules ( $\times 400$ ).

The histologic findings of interest were chiefly in the liver and kidneys. In the liver there was considerable disorganization of cell cords, marked cloudy swelling, some fatty change, and degenerative changes in the nuclei, with only a few scattered zones of actual necrosis (see Fig. 5). Numerous lymphocytes and plasma cells occurred in the periportal areas, but no connective tissue proliferation was noted. Considerable bile pigmentation was present. The kidneys showed fairly normal glomeruli, with both hematoxylin-eosin and azocarmine stains. Marked tubular degeneration was evident, with many areas in which groups of convoluted tubules were completely necrotic (see Fig. 6). Masses of bile pigment were scattered throughout the tubules. Very little iron pigment was shown in the Prussian blue stain. In the spleen there was a moderate increase of iron pigmentation. Bone marrow smears and sections were not remarkable. No organisms were seen in smears from the spleen, and cultures were sterile.

#### COMMENT

The histologic appearance of the liver is not that of acute yellow atrophy. It does show evidence of hepatic degeneration, but not as much as would be expected from the degree of impaired liver function demonstrated clinically. There is, however, no reason for believing, from either clinical or laboratory evidence, that hemolytic anemia was responsible for any appreciable part of

TABLE I

AUTOPSY NO.	CLINICAL DIAGNOSIS	CAUSE OF DEATH	TOTAL DOSAGE OF SULFA-NILAMIDE	DATES ADMINISTERED	DATE OF DEATH	SULFA-NILAMIDE IN BLOOD* MG./PER CENT	SULFA-NILAMIDE IN TISSUES† MG./PER CENT
21064	Pyelonephritis	Uremia	27 Gm.	Dec. 4-12 (1938)	Dec. 12 (1938)	---	8.4 liver
20105	Influenzal meningitis	Same	10.5 Gm.	June 8-11	June 12	---	12.0 liver
20927	Scarlet fever	Granulocytopenia	101 Gm.	Nov. 2-18	Nov. 25	5.5 (Nov. 17)	2.5 liver
20440	Puerperal sepsis	Streptococcus viridans septice-mia	46 Gm.	July 8-Aug. 10	Aug. 18	---	0 liver
19798	Thrombophlebitis erysipelas		24 Gm.	Mar. 24-April 1	April 13	---	0 liver
21055	Pneumococcal meningitis	Same	730 Gm.	Oct. 2-Dec. 4	Dec. 16	33 (Nov. 1) 15 (Dec. 12)	3.1 liver 2.7 kidney
19973	Gonorrheal arthritis	Hepatorenal syndrome	34 Gm.	May 6-11	May 16	0 (Post-mortem blood)	2 liver
DA 724	Streptococcus viridans subacute bacterial endocarditis	Same	24‡ Gm.	Jan. 10-15 (1939)	Jan. 16	---	3.9 liver 2.3 spleen 8.3 kidney

\*Determinations were by the method of Marshall for free sulfanilamide, and the value given is that last determined.

†Five grams of tissue were macerated with 10 c.c. of water and mixed with 5 c.c. of trichloroacetic acid. The free sulfanilamide content of the filtrate was determined.

‡This patient received sulfapyridine. The usual sulfanilamide standards were used in the determinations.

the patient's jaundice. The lesion in the kidney is more striking than that of the liver, and is in keeping with the patient's picture of progressive oliguria and failing kidney function. It suggests direct injury to the kidney, largely tubular, probably by the same agent that injured the liver, although it might conceivably be included in the category of "hepatorenal syndrome," with kidney damage dependent on the products of liver damage.

As in the previously reported cases, the cause of the injury to the liver and kidneys is not clear. The drug was administered to a patient not seriously ill, in an amount frequently used in clinical practice. Although sulfanilamide is acetylated by the liver and excreted by the kidney, neither the free nor conjugated form has been shown to be injurious to these organs by experimental methods. Hageman<sup>7</sup> reported no apparent damage to the liver and kidneys, and noted only excess deposits of iron-containing pigment in the spleen, after the administration of lethal doses to mice. It has, however, been noted that the bromsulphthalein excretory function of the liver is impaired in patients re-

ceiving sulfanilamide. It is also possible that chronic alcoholism may have predisposed the patient to hepatic damage.

Although the mechanism of liver and kidney damage is not understood, it is clear from experimental observation, that with conjugation impaired by liver damage and excretion impaired by kidney damage, retention of free sulfanilamide might occur in the tissues. It is also theoretically possible that the retention of the drug in a particular organ need not necessarily be reflected in the blood sulfanilamide level. We, therefore, thought it of interest to determine the tissue sulfanilamide content at autopsy. Since few figures are available on human tissues, control determinations were done on tissues from several other autopsies where sulfanilamide had been administered prior to death. The control observations are not very satisfactory because of the variation in amount of drug given, and in the period elapsing from cessation of administration to death. The results are given in Table I.

Marshall and collaborators<sup>28</sup> have shown that under normal conditions in experimental animals, free sulfanilamide is rather evenly distributed throughout most body tissues. Engelfried<sup>29</sup> recorded similar findings in human autopsy material from one patient. Greene and Hotz<sup>27</sup> noted considerable increase in the concentration of sulfanilamide in the liver as compared to the lung in a case of hepatic degeneration, apparently due to sulfanilamide.

Although only the liver was analyzed in our case (19973), it does not show an abnormally high value as compared with the findings in other cases at a similar period after administration of the drug. Further chemical studies of tissue at autopsy, especially where there have been toxic effects, may be of interest.

#### SUMMARY

Five deaths are reported following the administration of sulfonamide drugs, three from granulocytopenia, one from hemolytic anemia, and one from liver and kidney damage. In a patient receiving 101 Gm. of sulfanilamide for scarlet fever, and in another patient receiving 64 Gm. of sulfanilamide for streptococcal sore throat, fatal granulocytopenia developed. A third case of fatal granulocytopenia occurred in a patient who was given 88.5 Gm. of sulapyridine in the treatment of bacterial endocarditis. In two of these patients the bone marrow was examined at autopsy and showed maturation arrest of the myeloid series at the myelocyte level. A typical acute hemolytic anemia developed within three days in a patient treated for erysipelas with 25 Gm. of sulfanilamide. Interference with renal tubular function by precipitated hemoglobin derivatives was thought to be a major factor leading to death in this patient. The fifth case showed clinical evidence of severe liver and kidney damage developing after the administration of 34 Gm. of sulfanilamide for gonorrheal arthritis. Degeneration of liver cells and necrosis of renal tubular epithelium were found at autopsy.

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## NUTRITION AND NERVOUS EXCITABILITY\*

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IN STUDIES on basal metabolic rate of Chinese it was found that the relatively lower heat production of Chinese was probably due to their greater degree of relaxation. This conclusion was arrived at in the following way: It was assumed that since man's voluntary musculature is more or less relaxed when sleeping, one's basal metabolic rate should be equal during sleep, but different while awake, depending upon the individual's degree of muscular relaxation. To test this, the oxygen consumption of Chinese and Westerners was determined during both conditions. It was found that in most Chinese the basal metabolic rate did not change appreciably after they had fallen asleep, but that it dropped considerably in Westerners, with a relatively higher waking basal metabolic rate. Westerners with a low waking basal metabolic rate did not show such a drop while asleep (Necheles, 1928; Necheles, 1930; and Necheles and Loo, 1932).

It is believed that the Chinese consume less protein and protein of lower biologic value than the Westerner; this may explain indirectly their lower basal metabolic rate. It is well known, of course, that the specific dynamic action of proteins lasts only as long as their digestion products are absorbed from the intestines and that, therefore, the simple concept of a higher or lower specific dynamic action would not be sufficient to explain a higher or lower basal metabolic rate in the completely fasting condition. It was thought possible, however, that a diet high in proteins might lower the threshold of muscular as well as of nervous and of reflex excitability beyond the period of digestive absorption, thereby raising muscle tonus and number or extent of reflex movements, and consequently oxygen consumption. With this idea in view, experiments were performed by one of us (H. N.), together with Dr. Ernest DeVries (Department of Neurology, at the Peiping Union Medical College, China. Chinese subjects were employed, and the threshold excitability of the branch of the facial nerve supplying the risorius muscle, as well as the ulnar nerve in the elbow, and movement of the fifth finger were recorded. A clinical wall plate was employed, and the amount of current used was read in milliamperes. The indifferent electrode was held against the chest, while the different one was applied by hand to the marked nerve point. Both electrodes were soaked in saline solution. The kathodal closing contraction (K.C.C.), the anodal closure contraction (A.C.C.), and the kathodal opening contraction (K.O.C.) were determined. After a constant level of excita-

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bility had been established in fasting subjects, they were given, on different days, meals consisting of  $1\frac{1}{2}$  lb. of broiled lean meat, or 120 Gm. of butter on a slice of toast, or a carbohydrate meal consisting of a fruit pudding with sugar and glutinous rice. After the meal the nervous thresholds were determined in twenty-minute intervals, and the oxygen consumption and the carbon dioxide production were measured. The results were quite uniform and indicated that proportional to the rise of the specific dynamic action after a meat meal (but not as regularly or extensively after fat, carbohydrate, or fasting), the nervous thresholds would drop considerably and return to control values at about the same time as the oxygen consumption and the carbon dioxide production.

Since this phenomenon might have been caused by a variation in skin resistance following ingestion of meat, control experiments were performed in which, following the technique of Richter (1926), the epidermis above the nerve point on one side of the body was perforated with a surgical needle, and the different electrode applied to that spot; the contralateral side with nonperforated skin served as control. It was found that following this procedure the lowering of nerve thresholds following a protein meal was no longer detectable. This proved, indirectly, that the results obtained in the previous experiments had been due to changes in skin resistance rather than to variations in nervous thresholds. At this point the experiments had to be interrupted because one of us (H. N.) left China. They were taken up again in Chicago on white persons. The technique was basically the same, except that small zinc electrodes were fastened to the skin with collodion and adhesive, that Cambridge electrode jelly was used instead of saline moistened pads, and that a more sensitive amperemeter, a more accurate rheostat, and radio batteries as source of current were employed. To our surprise, results were entirely different from those found in Peiping. Seven male and 6 female persons were employed. It was found that the nervous threshold, when followed over prolonged control periods of fasting, showed rhythmic variations, and that after carbohydrate, fat, or protein meals no significant or constant variation in nervous threshold was found either on the unperforated or on the perforated skin.

#### DISCUSSION

The observations in China were made by two independent observers (Dr. DeVries and H. N.), both of whom obtained essentially the same results. The tests in Chicago were performed by one of us (L. M.) after having obtained proficiency in the technique used, and repeating each reading at least three times. We offer the following possible explanations of the discrepancy between our results on Chinese in Peiping and on white persons in Chicago: in case of the subjects in Peiping an unaccustomed high protein meal may have produced a change in skin resistance. Unfortunately, we were not able to obtain Chinese subjects in Chicago, although results might not be the same as in China because we know that the nutritional standards of the American Chinese are quite different from those in their homeland. The second possible explanation may be found in the fact that in the experiments done in Peiping,



the different electrode may not have been applied to the same spot at each test; this is not probable, however, in view of similar curves in a great number of tests done by two different observers. A third possible explanation may be seen in the difference in climate between Peiping and Chicago, and its effect on skin resistance.

A number of other experiments were performed in Chicago in order to analyze the relation between food substances and reflexes under more controlled conditions. In consequence of the negative results obtained, they will be mentioned only briefly:

1. Reflex time (Tuereck) in frogs before and after injection of, or perfusion with, amino acids in buffered solutions.

2. Height and reaction time of knee jerk and chronaxie of ipsilateral flexor and crossed extensor reflexes in anesthetized dogs before and after intravenous injection of amino acids.

3. Reaction time in man (Dunlop chronoscope) before and after carbohydrate, fat, or protein meals.

4. Height of knee jerk (mechanical hammer) in man before and after above meals, as well as before and after ingestion of amino acids.

#### SUMMARY

In order to explain the difference in basal metabolic rate between Chinese and Westerners, experiments were conducted in China and in the United States to determine the effect of various foods on the neuromuscular system. It was found that the skin resistance of Chinese in Peiping apparently decreased after a protein meal, while that of Westerners in Chicago did not.

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# THE INTRAVENOUS ADMINISTRATION OF SUCROSE SOLUTIONS AS A MEANS OF PRODUCING INTENSE DIURESIS\*

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IN THE acute stage of urinary infections the washing out of the urinary passages is one of the most effective means of treatment. The use of large amounts of fluid by mouth or by other routes is often sufficient to bring about a flow of urine. In some cases, however, this alone is not sufficient and a more powerful diuretic is necessary. It is our object to find out what solution can be given with a minimum of harm to the tissues and yet produce a maximal diuresis.

From former studies on the excretion of bacteria after the injection of 20 per cent sucrose solution, it was shown that a sufficiently intense diuresis could be produced so that one-half of the cultures of 0.5 c.c. of urine were sterile, whereas before the diuresis was started the urine in the same quantities contained innumerable bacteria.<sup>1</sup> This observation led us to investigate the value of sucrose as a diuretic and to find out its toxicity and its relative value as compared with dextrose, sorbitol, urea, and sodium sulfate.

For a comparison of dextrose and sucrose as safe diuretic agents, there were available experiments carried out some years ago on rabbits. Thirteen rabbits were injected with 20 per cent solution of dextrose, varying in amounts from 60 to 108 c.c. per kilogram of body weight per hour, and 16 animals were injected with 20 per cent solution of sucrose, varying in amounts from 107 to 167 c.c. per kilogram per hour. The average volume of urinary output of the 13 animals receiving dextrose was 73 per cent of the volume injected, and for the 16 animals receiving sucrose the output was 94 per cent. This would indicate that even though the tonicity of the dextrose solution was practically twice that of the sucrose, its effect was 21 per cent less. When the quantities injected are taken into account, this difference is very much greater because much larger amounts of sucrose were injected, as is seen in Table I. The greater danger of these injections of dextrose, although much smaller in amount than the sucrose injections, is seen from the death of 92 per cent of the animals that received dextrose in contrast to the death of 25 per cent of the animals that received a 65 per cent greater volume of 20 per cent sucrose solution. Thus it is evident that sucrose solution is both better and safer than dextrose solution for purposes of diuresis when large amounts are to be injected in a short period (Table I).

In our experiments with 20 per cent solution of sucrose we found that the maximum diuresis in one hour was obtained without subsequent mortality when 100 c.c. for each kilogram of body weight was injected in one hour. Compari-

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sons were made of the diuretic and lethal effect of equal volumes (100 c.c. per kilogram) of solutions of equal osmotic pressure of sodium sulfate, sorbitol, and urea, the latter dissolved in physiologic salt solution.

TABLE I

A COMPARISON BETWEEN THE TOXICITY OF A SINGLE LARGE INJECTION OF SUCROSE AND A SINGLE LARGE INJECTION OF DEXTROSE

	16 ANIMALS INJECTED WITH 20% SUCROSE Average Injection 140 c.c. per kg. in 1 Hour	13 ANIMALS INJECTED WITH 20% DEXTROSE Average Injection 85 c.c. per kg. in 1 Hour
Died within 1 hour	6.2%	69.2%
Died within 24 hours	25.0%	92.3%
Recovered	75.0%	7.7%

*The Diuretic Effect of 2.8 Per Cent Solution of Sodium Sulfate When Administered Intravenously in a Dose of 100 c.c. per Kilogram per Hour.*—The animals did not tolerate the solution well, so that injections were given only three times a week rather than five times a week as were the sucrose injections. The urinary output for every 100 c.c. injected was 85 c.c. for a total of eighty-three injections in three animals. The two animals that received thirty-two and thirty-four injections, respectively, died\*. The one animal that received only seventeen injections recovered. The phenolsulfonphthalein output ranged in the animals between 57 and 100 per cent. The changes in the kidneys of the two animals receiving the large number of injections were relatively mild as compared with those in similar animals receiving sucrose. The tubular epithelium was slightly swollen and vacuolated, but otherwise there were no changes in the kidneys.

*The Diuretic Effect of 3.5 Per Cent Urea Solution in Normal Saline When Administered Intravenously in a Dose of 100 c.c. per Kilogram per Hour for One Hour.*—The administration of urea in water produced hemoglobinuria, so that urea was administered in normal saline solution. This solution was well tolerated. Six animals were injected, one animal receiving sixteen injections, another receiving twenty-six injections, and the rest a smaller number of injections. These animals received three injections a week. The diuresis was 73 c.c. per hour for each 100 c.c. per kilogram per hour injected. The animal receiving the twenty-six injections was killed twenty-four hours after its last injection. At necropsy the kidneys were not enlarged and appeared normal on section. Microscopically the convoluted tubular epithelium was swollen, the protoplasm granular and full of vacuoles. Many tubules were filled with granular debris. Some tubules were lined with narrow cells which had a shredded border toward the lumen. The findings resembled somewhat those found on injections of sucrose, except that the swelling of the cells was not so great.

*The Diuretic Effect of 11.7 Per Cent Solution of Sorbitol When Administered Intravenously in a Dosage of 100 c.c. Per Kilogram Per Hour for One Hour.*—Recently suggested for its dehydrating effect, it was thought that sorbitol might have a diuretic effect superior to that of sucrose. Single experi-

\*Four other rabbits died within eighteen hours after receiving one, one, two, and four injections, respectively.

ments on two rabbits showed in one case an excretion of 80 c.c. and in the other 90 c.c. of urine for each 100 c.c. injected.

TABLE II

AVERAGE OUTPUT IN ONE HOUR DURING INJECTIONS OF 100 C.C. PER KG. PER HOUR OF ISOTONIC SOLUTIONS OF SODIUM SULFATE, UREA, AND SUCROSE

NATURE OF SOLUTION INJECTED	URINARY OUTPUT, C.C. PER KG.	NUMBER OF INJECTIONS
2.8% solution of sodium sulfate	85	83
3.5% solution of urea	73	42
20% solution of sucrose	131	33

## EFFECT OF INJECTIONS OF SUCROSE

The comparison of the effectiveness of sodium sulfate, urea, and sucrose as diuretics is seen in Table II. It will be noted that in the special series of thirty-three injections of sucrose, 131 c.c. of urine per kilogram were returned within the hour when 100 c.c. of 20 per cent solution of sucrose per kilogram had been injected intravenously. This was the only one of the substances in which more was returned in the hour than was injected. We found the sucrose to be safer than the sodium sulfate which gave the next best results. It is also of interest to compare the percentage of excretion of urine when 100, 75, and 50 c.c. of 20 per cent solution of sucrose for each kilogram of body weight was injected within an hour. The figures were very close—131, 136, and 136 per cent of the amounts injected. It would seem that a 20 per cent solution of sucrose was the most effective, and under these circumstances the safest, as well as a very inexpensive, diuretic solution for intravenous use.

Early in our experiments it was evident that the intravenous injection of 20 per cent solution of sucrose produced extreme hydropic degeneration of the convoluted tubules, and when large amounts were injected, it produced a lowering of the phenolsulfonphthalein output and subsequently a urinary output that fell to zero. It was necessary, therefore, to determine whether these changes in the convoluted tubules were of a permanent or of a transient character and whether functionally the kidney showed permanent damage which would contraindicate the use of sucrose. In this connection we considered the possibility of functional incapacity of the convoluted tubules that might shed some light on their secretory activity, particularly with regard to reabsorption of water from the glomerular filtrate. Uniformly, however, the concentration of the urine following the injection indicated that in spite of the marked hydropic degeneration of the tubular epithelium this function remained intact. We, therefore, gave a number of animals repeated injections of 20 per cent solution of sucrose.

Of the animals injected we want to mention only three that received the greatest number of injections, one receiving thirty-eight, a second receiving fifty-two, and a third which received sixty-eight injections of 20 per cent sucrose in distilled water in one-hour periods. The essential data concerning these animals are given in Table III. The animals remained in good condition throughout the course of the experiments; rabbit 2 gave birth to six young twenty-five days after the last injection of sucrose. Attention is called to the maintenance

of good diuretic response to the injection of sucrose and also the phenolsulfonphthalein excretion which was not greatly impaired by the repeated injections.

TABLE III

EFFECT OF REPEATED INJECTIONS OF 20 PER CENT SOLUTION OF SUCROSE ON THE FUNCTION OF THE KIDNEY

NUMBER OF INJECTIONS	AMOUNT INTRA- VENOUSLY INJECTED (C.C.) PER KILOGRAM PER HOUR	URINARY OUTPUT (C.C.) PER KILOGRAM PER HOUR	PERCENTAGE EXCRETION PHENOLSULFON- PHTHALEIN
Rabbit 1, weight 2.3 kg., receiving 38 injections in 82 days.			
1	50	46	56
2	75	105	63
1. Left nephrectomy 3 days after 6th injection. Injections resumed after 23 days.			
12	50	42	44
11	75	66	40
9	100	63	48
2. Biopsy of right kidney 5 days after 38th injection			
Rabbit 2, weight 2.4 kg., receiving 52 injections in 71 days.			
1	100	132	66
12	50	76	52
2	75	126	33
22	100	118	54
1. Left nephrectomy 1 day after 37th injection. Injections resumed after 5 days.			
15	50	50	44
2. Biopsy of right kidney 4, 39, and 105 days after 52nd injection.			
Rabbit 3, weight 2.0 kg., receiving 68 injections in 110 days.			
4	50	35	62
8	75	91	54
9	100	98	35
1. Left nephrectomy 3 days after 21st injection. Injections resumed after 8 days.			
10	50	46	34
10	75	57	30
12	100	59	25
2. Biopsy of right kidney 1 day after 53rd injection. Injections resumed after 5 days.			
15	50	49	28
3. Biopsy of right kidney 39 and 105 days after 68th injection.			

The kidneys and sections removed one to five days after several injections of sucrose appeared swollen with the cortex thickened. The convoluted tubules were greatly swollen; the epithelium of the convoluted tubules was large and clear, so that the cells resembled those of hypernephroma. The nuclei were small and pyknotic, and many cells did not appear to have nuclei. The glomeruli and the other structures of the kidney appeared to be normal. Sections removed one to five days after the last injection appeared similar, except that in the latter an increased staining of some regions of the convoluted tubules suggested an increase in the protoplasm of the swollen cells.

Fig. 1 illustrates the condition of the convoluted epithelium one day after the fifty-third injection of sucrose. Very few cell outlines could be made out. In portions of tubules only small shreds of protoplasm were seen and to a considerable extent the cells lining the tubules did not show nuclei. When cell out-

lines were distinguishable, the tubules were practically swollen shut. In some regions where cell outlines were represented only by small masses of granular material, the lumen of the tubules seemed large. The glomerular capsules were distended, so that there was much space about the glomerulus.

Sections taken thirty-nine days after the sixty-eighth injection indicated almost complete recovery from what seemed, in the sections taken within a few days after injection, to be irreparable damage to the convoluted tubules. The sections appear like those from the normal kidney of the rabbit, except perhaps that the cells of the convoluted tubules were slightly larger (Fig. 2). Sections taken one hundred and five days after the last injection were entirely indistinguishable from sections of normal kidneys.

*Injection for Six Hours.*—Having convinced ourselves that sucrose solution even in 20 per cent concentration injected five times a week for a number of weeks does not result in any permanent damage to the kidney and does not seriously interfere with its function, it next seemed indicated to continue the set rate of injection of 100 c.c. per kilogram per hour for longer periods. In extending the time, we are putting a much greater strain on the circulation as well as the kidney, and it is essential, in order to be successful, that there be a balance between intake and outgo. Former experiments had shown us that even when 50 per cent sucrose was injected, the kidney could not put out a concentration of sucrose of more than 10 per cent. As a result of a one-hour experiment we were pretty thoroughly convinced that sucrose is the best diuretic for intravenous use, but we felt that a few six-hour experiments with the various diuretics used in the one-hour experiments would be of confirmatory value.

Our first injections with 8 and 10 per cent sucrose solution in water showed us that the good diuresis of the early hours rapidly disappeared with a diminishing output of sodium chloride. The chloride concentration, as well as the volume of urine, was greatly reduced, and there was a retention of sucrose proportional to the water retention. Two of the three animals died, and the output for the six-hour period was only 66, 44, and 39 per cent of the amount injected.

To correct this lack of sodium chloride, as well as a lack of potassium and calcium, one-half strength Ringer's solution was used because it is approximately equivalent to the amount of inorganic salts excreted in the first hour of marked diuresis with sucrose in water. Using 8 per cent sucrose in one-half strength Ringer's solution, there was a surprising improvement in the output of urine—an average output of 88 per cent of the volume injected—and there was no definite retention of water, sucrose, or sodium chloride. The general condition of these animals at the end of the injection was practically normal. Not one of the eight injected animals died. One animal had two additional six-hour injections in the course of two months, excreting 92 per cent and 95 per cent of the amount injected the second and third times, and one animal had two injections in a period of a month.

Ringer's solution was injected at the same rate of 100 c.c. per kilogram per hour for six hours in five animals, one of which died during the course of the fifth hour. In all there was a marked retention of water and salt.

Three animals receiving 1.44 per cent urea in one-half strength Ringer's solution died within forty-eight hours, one dying after two and one-half hours



Fig. 1.—Section of kidney ( $\times 140$ ) removed from rabbit 3 twenty-four hours after the fifty-third injection of 20 per cent sucrose.

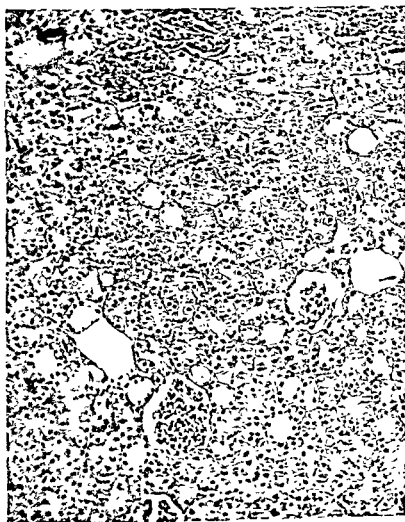


Fig. 2.—Section of kidney ( $\times 140$ ) removed from rabbit 3 thirty-nine days after the sixty-eighth injection of 20 per cent sucrose.

of injection. Each showed a marked retention of water, urea, and sodium chloride.

Three animals received 1.55 per cent sodium sulfate in one-half strength Ringer's solution; one died four hours after the injection, and one within forty-eight hours. Each animal showed retention of water, sulfate, and sodium chloride during the injection period.

One of the four animals receiving 4.2 per cent dextrose in one-half strength Ringer's solution died at the end of four hours, and all showed retention of water and sodium chloride.

The two animals receiving 4.5 per cent sorbitol in one-half strength Ringer's solution lived and excreted 48 per cent of the volume injected, retaining sodium chloride proportional to the water retained. Five animals received 6 per cent sorbitol in the same solution and put out on an average 80 per cent, and three of the animals died within forty-eight hours.

TABLE IV

DIURESIS AFTER INJECTION OF 100 C.C. PER KILOGRAM PER HOUR FOR SIX HOURS

SOLUTION	PERCENTAGE OF INJECTED SOLUTION EXCRETED	NUMBER OF ANIMALS INJECTED	NUMBER OF ANIMALS WHICH DIED WITHIN 48 HOURS
1.44% urea in $\frac{1}{2}$ Ringer's solution	38	3	3
Ringer's solution	47	5	1
4.5% sorbitol in $\frac{1}{2}$ Ringer's solution	48	2	0
4.2% dextrose in $\frac{1}{2}$ Ringer's solution	62	4	1
1.55% sodium sulfate in $\frac{1}{2}$ Ringer's solution	73	3	2
6% sorbitol in $\frac{1}{2}$ Ringer's solution	80	5	3
8% sucrose in $\frac{1}{2}$ Ringer's solution	88	8	0

From Table IV it is to be seen that an 8 per cent sucrose solution in one-half strength Ringer's solution produces a fair balance of fluid, sodium chloride, and sucrose. As was to be anticipated the changes in the kidneys after a twenty-four-hour and a forty-eight-hour interval resemble very closely the changes in those experiments in which a 20 per cent sucrose solution was injected for one hour and the animal killed after twenty-four and forty-eight hours. These changes, however, did not interfere with the function of the kidney sufficiently to prevent an animal tolerating three such injections and at the last injection putting out a greater volume than in the previous one.

None of these eight animals died as a result of the injections, even when two had multiple injections. This, taken in conjunction with a higher percentage excretion of the sucrose-injected animals, makes it evident that our evaluation of sucrose as the best diuretic by the one-hour test is also borne out by the six-hour experiments.

It can thus be definitely stated that a rabbit receiving 100 c.c. per kilogram per hour of 8 per cent sucrose in one-half strength Ringer's solution for six hours will excrete almost 90 per cent of the volume injected without serious danger to life. This represents more than half of his body weight in fluid excreted in six hours. We realize that the solutions used in these experiments



are still merely rough approximations of the ideal diuretic solution and that many experiments on different animals will have to be tried before we shall know the ideal diuretic for man.

#### SUMMARY

Diuresis may be obtained in rabbits by the intravenous injection of 20 per cent solutions of sucrose. One hundred cubic centimeters of this solution for each kilogram of body weight injected in one hour produced an excretion of urine approximately 130 per cent of the volume injected. Marked hydropic degeneration of the convoluted tubules occurred, and in the next twenty-four to forty-eight hours there was a marked lowering of the phenolsulfonphthalein output and temporary anuria occasionally developed. That these changes produced no permanent damage was demonstrated by repeated injections five times each week for as many as sixty-eight injections. Diuresis was unimpaired, and even though a section of the kidney removed a few days after many injections appeared irreparably damaged, within thirty-nine days the kidney on removal appeared normal grossly and microscopically and could not be distinguished from a normal kidney.

Sodium sulfate, urea, dextrose, and sorbitol injected in a similar manner in solutions with tonicity equivalent to 20 per cent sucrose produced less diuresis in every case. The histologic changes produced in the kidneys were similar in nature but definitely less than those produced by sucrose. With the exception of sorbitol all were definitely more toxic and could not be injected repeatedly as often as sucrose.

An intense diuresis was maintained for six-hour periods by the continuous intravenous injection of solutions. With sucrose solutions urine equivalent to one-half of the weight of the animal was excreted in the six-hour period. To avoid lethal effects and to maintain the excessive flow of urine it was found necessary to maintain a balance of the solution injected and the urine excreted with reference to water, sucrose, and salt. This was accomplished with a solution of 8 per cent sucrose in one-half strength Ringer's solution.

Solutions of urea, sodium sulfate, and dextrose, of tonicity equivalent to 8 per cent sucrose, in one-half strength Ringer's solution were lethal or definitely endangered the life of the animals. These solutions, as well as an equivalent sorbitol solution, produced less diuresis and permitted a greater retention of water and sodium chloride than did sucrose. Greater concentration of sorbitol produced diuresis approaching that of 8 per cent sucrose, but was fatal to three of five animals used.

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# INTERRELATION BETWEEN THE VITAMIN B COMPLEX AND THE ANTERIOR LOBE OF THE PITUITARY GLAND\*

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THE work of Spies and others has demonstrated the specificity of nicotinic acid in the treatment of pellagra, but despite this there are some patients who fail to respond to large doses of nicotinic acid even when it is combined with liver extract and an adequate diet.

As a result of the treatment of two patients<sup>1</sup> reported as cachexia due to deficiency of the anterior lobe of the pituitary gland, it was noticed that pellagrous lesions improved although they had failed to improve with specific therapy.

## CASE HISTORIES

CASE 1.—L. W., a 64-year-old white laborer, entered Cook County Hospital on December 26, 1937.

His immediate complaints were diarrhea, loss of weight, and edema of the feet. He was irritable and after a few days left the hospital, returning January 27, 1938 with the same complaints. He stated that a gastro-enterostomy had been done in 1915, although no evidence of such an operation can be found by either direct gastroscopy or x-rays of the stomach.

The edema of the feet had been present at various times during the past three years, and recently he had been treated at a dispensary for a cardiac condition. The remainder of the history was irrelevant.

At entrance the physical examination revealed an emaciated, weak, but not acutely ill patient. His temperature was 98.2° F., pulse 48, respirations 9 per minute, and blood pressure 126/80. An acute glossitis was noted. Examination of the chest and abdomen was negative. A gastroscopic examination revealed a reddened mucosa with a few erosions. The proctoscopic examination showed a reddened mucosa. The spinal and blood Wassermann tests were negative. The basal metabolic rate was -34. The blood chemistry showed total nonprotein nitrogen 25; urea 15.41, uric acid 2.5, cholesterol 160, sugar 86, total proteins 6.83, albumin 4.37, globulin 2.46, and ratio 1.77. There was a complete achlorhydria after an Ewald meal.

The diarrhea persisted, with five to twelve stools daily. The feces were yellow, watery, with a low specific gravity, and contained no blood. No parasites were found.

Neither the glossitis nor the diarrhea was improved by large doses of nicotinic acid, parenteral administration of liver extract, and an adequate diet. He was also treated with thyroid extract and insulin.

He lost weight (from a normal of 180 pounds to 89 pounds) and became helpless from weakness.

On October 19, 1938, daily intramuscular injections of an extract of the anterior lobe of the pituitary gland† were begun on a suspected diagnosis of a pituitary cachexia.

\*From the Department of Medicine, Northwestern University Medical School and the Robert Bruce Preble Research Laboratory of the Cook County Hospital, Chicago.  
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†The extract of the anterior lobe of the pituitary gland, "polyansyn," of Armour and Company was used. The method of preparation of polyansyn should destroy all water-soluble vitamins.

Shortly after beginning the administration of the extract of the anterior lobe of the pituitary gland, the diarrhea improved, the glossitis disappeared, and after thirty days a gain in weight and strength was apparent. By May, 1939, six months later, he weighed 167 pounds and his basal metabolic rate was -10. The pituitary extract was discontinued in May, 1939. He continued well until October 15, 1939. After a recurrence of the diarrhea and a loss of 9 pounds, 1 c.c. of the gonadotropic hormone of the pituitary gland was given intramuscularly for thirty days. The diarrhea and weight loss were promptly relieved. The gonadotropic hormone was discontinued because of the belief that it is impossible to procure an extract free of the other hormones. He continued well until April, 1940, when the diarrhea returned and later a severe glossitis.

He was allowed to lose 13 pounds after which 1 c.c. of the polyansyn was injected daily. Within forty-eight hours the diarrhea ceased, and the tongue was no longer painful. The redness disappeared in three days.

CASE 2.—Mrs. T. R., a white woman 44 years old, was first seen on August 14, 1939.

Her initial complaints were weakness, loss of weight, and diarrhea. The diarrhea had been present at intervals for the past fifteen years, and continuous with several watery stools daily for the past five years.

The essentials of examination were emaciation (weight 92 pounds), a peculiar wizened facies like that seen in hypopituitary dwarfism, and a slight glossitis.

The blood examination showed hemoglobin 14.5 Gm., red blood cells 4,800,000, white blood cells 6,050, total nonprotein nitrogen 29.5, glucose 91, cholesterol 213, and chlorides 485. The basal metabolic rate was -7. Analysis of an Ewald meal revealed free hydrochloric acid 6, total acidity 22. X-ray examination of the gastrointestinal tract was entirely negative. There was a trace of blood in the stools on one occasion. No amoebae were found, although a single cyst had been reported a year previously. Because of the possibility that this was a case of atypical Simmonds' disease, she was given 1 c.c. of polyansyn intramuscularly daily. The glossitis promptly disappeared and the diarrhea improved. By February, 1940, she had gained 13 pounds, the most she has ever weighed.

The patient has since then become less emotional, is able to take care of a four-room apartment, and resume normal social activities. Her menstrual periods are less painful and have a more normal flow. Nevertheless, during each menstrual period and the week thereafter there is a moderate to severe return of the diarrhea. Recently 2 c.c. of polyansyn daily during the week preceding the menstrual period greatly decreased these recurrent diarrheas. If the polyansyn is stopped, the symptoms return within two weeks.

In view of the prompt recovery of the glossitis following the use of an extract of the anterior lobe of the pituitary gland, it appeared rational to apply this therapy to persons with pellagra who had failed to respond to the administration of large amounts of nicotinic acid, riboflavin, liver parenterally, and adequate diets.

CASE 3.—C. N., a white male laborer, aged 49 years, entered the Cook County Hospital on August 29, 1939.

His immediate complaints were epigastric pain for the past year, loss of appetite for six months, and a loss of 50 pounds during the past year.

The epigastric pain had no relation to meals, although he occasionally vomited about one hour after a meal. He had a diarrhea of four to five liquid stools daily.

The essential physical findings were emaciation, a severe glossitis (a red beefy tongue), and a sore reddened pharynx.

The results of laboratory examinations showed that the urine contained a two-plus sugar and a three-plus albumin. An Ewald meal revealed an achlorhydria. No parasites nor blood were found in the feces.

A glucose tolerance test showed fasting glucose 106; at 9 A.M., 272; 10 A.M., 200; 11 A.M., 158.

The blood examination revealed hemoglobin 10 Gm., red blood cells 3,590,000, white blood cells 4,800, total nonprotein nitrogen 23, cholesterol 135, cholesterol esters 36, and phosphatase 7.38. The Kahn reaction was negative. The basal metabolic rate was -11.

X-ray of the gastrointestinal tract revealed no abnormality. A gastroscopic examination revealed a red and swollen mucosa.

On September 13, 1939, an erythematous eruption was observed on the face and in the groin. There was also maceration of the scrotum and the thighs. At this time 600 mg. of nicotinic acid daily, dilute hydrochloric acid, and insulin for the diabetes were instituted. The skin and mouth lesions showed no improvement, and on October 17, 1939, the patient was referred to the outpatient diabetic and skin clinics.

He re-entered the hospital on December 12, 1939, with typical pellagra lesions of the tongue, face, cracked scaling lesions of the lips, and corners of the mouth, symmetrical eruptions on the elbows, hands, and feet, and the groin and perianal regions. The diarrhea had become much worse. He was given 800 mg. of nicotinic acid on entrance and then 400 mg. daily until January 30, 1940. Ninety-nine milligrams of riboflavin daily were given for one week. In the meantime a positive x-ray and gastroscopic diagnosis of carcinoma of the stomach were made and all treatment was stopped.

On February 13, 1940, he was referred to our service for a trial of polyansyn. On his date the lesions were as described on December 12, 1940, and, in addition, a severe excoriation of the scrotum was found.

The diarrhea stopped after 8 c.c. of polyansyn was injected intramuscularly in daily doses of 2 c.c. At this time the patient was completely bedridden. In fourteen days he was able to walk. The scrotal, perianal, and hand and feet lesions cleared completely. The scaling of the face disappeared, but the lesions at the angles of the mouth improved about 80 per cent and the tongue about 65 per cent. The cessation of polyansyn for two to three days caused the prompt reappearance of diarrhea, glossitis, and lip lesions and the anal-scrotal and perineal lesions a few days later. One cubic centimeter of polyansyn will stop the diarrhea in twenty-four hours.

The patient has had no evidence of diabetes for the past three months. Although he is not entirely relieved, the fully 80 per cent improvement has added greatly to his comfort.

CASE 4.—Mrs. T., aged 44 years, a white waitress, was first seen in the Northwestern University hematology clinic by Doctors Howard Alt and Richard Young on December 6, 1937.

Her complaint at that time was a diffuse swelling of the right parotid gland, redness and soreness of the mouth and tongue, and painful fissures at the angles of the mouth. She had complained of vomiting and belching of gas for fifteen years prior to her admission to the clinic. In 1927 she was examined at the Mayo Clinic.

No organic disease was found and it was then suggested that she had a vitamin deficiency.

Until November, 1939, she was treated for a secondary anemia and a vitamin deficiency. Iron, yeast, meat, oranges, and grapefruit were added to a supposed liberal diet, but her low income prevented her strict adherence to it. From November 21, 1939, 5 mg. of riboflavin and 300 mg. of nicotinic acid daily were also given. On January 6, 1940, the nicotinic acid was increased to 900 mg. daily for two weeks. From March 6 to March 16, 1940, 20 Gm. daily of ventriculin were given. On March 16, 1940, she was referred to us. At that time her tongue was red and painful. She had painful cracks at the corners of her mouth, with scaling of the skin at the lip borders. These lesions had not completely healed in three years.

She was given 2 c.c. of polyansyn daily. At the end of five days the mouth was entirely healed and the tongue was less red and painful. She continued well with 10 c.c. of polyansyn weekly until one week before her menses, when the angles of her mouth once more became sore and cracked, and her tongue became red and painful. The injections were continued and after menstruation ceased, the lesions promptly disappeared.

With the next menses the angles of the mouth became painful but did not crack. The condition disappeared before the menses were completed, and the menses became less painful during treatment. When last seen, May 10, 1940, she was entirely well.

CASE 5.—C. F., a Chinese male, 54 years old, entered the Cook County Hospital with a moderate cardiac decompensation, due to an aortic stenosis of rheumatic origin.

He showed no other significant findings, except a symmetrical dry scaling eruption on the backs of the hands, the buttocks, and the thighs. These lesions were diagnosed as pellagra. He was treated for his cardiac condition but kept on a vitamin-poor diet for ten days. At



PLATE I. Case 4.—Before treatment with polyansyn. Note cheilosis lesion in angle of the mouth.



PLATE II. Case 4.—After treatment with polyansyn.



the suggestion of Dr. Dunbar he was given 1,000 c.c. of glucose intravenously on March 20. The next day all the lesions were acutely inflamed and painful, and the backs of his hands were cracked and exuded serum.

He was continued on the low vitamin diet and given 2 c.c. of polyansyn daily. Improvement was prompt, and on May 9 all evidence of scaling was gone.

#### DISCUSSION

With the exception of Case 5, all patients had been well treated as pellagrins and yet showed little or no improvement for long periods of time. The use of an extract of the anterior lobe of the pituitary gland, as already stated, was suggested by the prompt improvement of the glossitis and diarrhea in Cases 1 and 2. These cases were considered as probably pituitary cachexias. The other cases had no evidence of pituitary hypofunction and yet improved with the administration of polyansyn. The lesions improved by the use of polyansyn are the same as those accepted as being due to deficiency of nicotinic acid and riboflavin. There is some evidence that starvation or vitamin B deficiency may lead to damage of the anterior lobe of the pituitary gland.

Marburg and Wenchebach observed necrotic areas in the anterior lobe of the pituitary glands of patients dying from beriberi.

Burke and McIntyre<sup>3</sup> found that rats on a thiamin-deficient diet did not lose weight as rapidly when given a growth-promoting extract of the pituitary as did those who did not receive the extract; they concluded that the growth-promoting hormone modifies the utilization of thiamin.

Brenneman<sup>4</sup> found that when day-old chicks were fed a standard diet only on alternate days growth was retarded to about 50 per cent of that of normal controls fed the same diet every day. The testes were lighter in the starved chicks. Male hormone limited the growth of the testes of the control chicks but not of the starved chicks. This observation is interpreted as evidence of decreased production of the gonadotropic hormone. This is further suggested by the fact that injection of an extract of the anterior lobe of the pituitary gland produces greater growth of the testes and combs in the starved chicks than in normal controls.

Sommer<sup>5</sup> found that women who had passed through puberty during the starvation caused by the blockade of Germany during 1914 to 1918 showed a high incidence of abortions and stillbirths. Delayed menstruation, amenorrhea, infantilism, and sterility were common. Corpus luteum hormones failed to cause improvement. Prolan benefited many cases. Greatly diminished lactation was common and was greatly improved by the use of lactogenic hormone. From these observations he concluded that the lactogenic and gonadotropic hormones of the pituitary gland were diminished by starvation.

Hundhausen<sup>6</sup> found that deprivation of vitamin B<sub>1</sub> caused a decrease in the thyrotropic and gonadotropic hormones in rats.

From this experimental work it appears that vitamin deficiency or partial starvation may cause a decrease of the hormones of the anterior lobe of the pituitary. This may serve to explain the results in all cases except Case 5. In this case, recovery occurred with the administration of polyansyn, although a low vitamin diet was continued. An attempt to answer some of these questions is now being done through a series of laboratory observations.

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## CONCLUSION

Evidence is presented indicating that an extract of the anterior lobe of the pituitary gland will cause recovery of pellagra lesions when they have failed to respond to nicotinic acid, riboflavin, parenteral liver, and adequate diet.

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## LABORATORY METHODS

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### MODIFICATION OF THE GALACTOSE TOLERANCE TEST BASED ON THE DIFFERENTIAL FERMENTATION OF GLUCOSE OCCURRING WITH GALACTOSE IN URINE\*

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THE galactose tolerance test continues to be used as a standard diagnostic procedure in cases of jaundice since its introduction by Bauer.<sup>1</sup> Few of the many tests for the study of liver function that have since been introduced enjoy such wide application. The diagnostic value and the simplicity of the procedure account for its widespread routine use.

It has been repeatedly affirmed that this test is of limited value if applied as a quantitative test of liver function (Lichtman, 1931<sup>2</sup>; Shay and Schloss, 1931, 1932<sup>3</sup>). It is, however, valuable in the differential diagnosis of jaundice. The procedure has been modified with the aim of increasing its diagnostic value. Thus galactose has also been estimated in the blood after either oral or intravenous administration of galactose (Jankelson, 1937<sup>4</sup>). Recently, Shay and Fieman (1937)<sup>5</sup> have also recommended a modification of the routine technique: differential fermentation of the urine for galactose and glucose. This addition seemed especially advantageous to them in the performance of the galactose tolerance test in patients with disturbed endocrine states, i.e., thyroid, adrenal, pituitary, and diabetes, where false positives might occur due to associated disturbances in glucose tolerance.

I have for some time contemplated the routine fermentation of the individual fractions after the oral administration of galactose. Bauer<sup>1</sup> originally attempted to identify the reducing substance in the urine as galactose after feeding this sugar. He applied the standard mucic acid test for this sugar to this purpose. This method, however, permitted a maximum recovery of only 70 per cent of galactose added to urine. Thus, part of the remaining 30 per cent of reducing substance in the urine after galactose feeding might possibly occur in the form of a sugar other than galactose. He discarded fermentation of the urine by yeast for the purpose of identification because of equivocal results he obtained in yeast fermentation of galactose.

The nature of the reducing substance which appears in the urine following the oral administration of galactose under pathologic conditions merits further study. Studies on the metabolism of galactose have indicated a conversion to glycogen both in animals and man (Roe, Gilman, and Cowgill, 1934;<sup>7</sup> Shay, Schloss, and Bell, 1931<sup>8</sup>). A rise in blood glucose may follow the ad-

\*From the Laboratories and the Medical Division, Service of Dr. George Baehr, the Mount Sinai Hospital, New York.

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ministration of galactose (Blanco, 1928;<sup>9</sup> Block and Weiss, 1930;<sup>10</sup> Harding and Grant, 1933<sup>11</sup>). Furthermore, in diabetes, blood glucose was found by Roe and Schwartzman (1932)<sup>12</sup> to be increased after galactose ingestion. Roe and Cowgill (1935)<sup>13</sup> noted a fall in blood galactose concomitantly with a gradual steady increase in blood glucose in rabbits following galactose administration. Thus, there is ground for the suspicion that under certain pathologic conditions the rate of the conversion of galactose to glucose and the tolerance for glucose may be impaired, so that some glucose may appear in the urine besides galactose.

It would be inadvisable to introduce any modification of the original technique which would change it from a convenient routine laboratory procedure into a complicated one. The differential fermentation of sugar, fortunately, is a simple procedure and requires no special apparatus or technique. The modification of the technique here proposed guards against possible sources of error. The problem involves a knowledge of the behavior of the yeast cell in relation to mixtures of fermentable and nonfermentable sugars. Under ordinary conditions baker's yeast does not ferment galactose.\* If galactose disappears from a solution after contact with a yeast suspension, phenomena other than fermentation, such as adsorption and diffusion, must be suspected. Sobotka, Holzman, and Reiner (1936)<sup>14</sup> have noted different rates of diffusion of two components of a sugar mixture. According to the speed of diffusion into the yeast cell, the sugars may be arranged in the following order: xylose > glucose > galactose > lactose. Sugars may leave solutions by diffusion into yeast cells without actual fermentation, as this occurs even when alcoholic fermentation is suppressed (by toxic agents).

While small amounts of a fermentable sugar, assumed to be glucose, may appear in the urine after galactose administration, according to the results of the present study, an error may arise if the diffusion of galactose into yeast cells in the fermentation of galactose-glucose mixtures is not first taken into account. Another source of error lies in the calculation when the reduction factor for galactose\* is applied to a fraction which is actually glucose.

#### PROCEDURE

The standard technique, as described by Bauer, was followed. The fractions were tested qualitatively for copper reduction and were then estimated quantitatively with Benedict's or Rudisch's solution.†

The latter solution boils with a minimum of bumping. The standard correction factor of 0.77 was applied for galactose. Fresh baker's yeast was employed. The yeast was washed if the supernatant contained a reducing substance. It is necessary to limit the amount of yeast to 5 per cent by volume of the solution to be fermented. Approximately equal amounts of yeast were used in all the fermentations which were carried on at temperatures between 40° and 45° C. for a period of sixty minutes. Controls consisted of a 1

\*Only after prolonged contact may baker's yeast be adapted to the fermentation of galactose.

†An alkaline copper solution recommended by Rudisch for the quantitative estimation of glucose in urine (International Contributions to Medical Literature, Festschrift in honor of Dr. A. Jacobi, 1900).

per cent solution of glucose in distilled water and a solution of between 1 and 2 per cent of galactose in normal urine, free of glucose. Sugar estimations were made on the supernatant liquid after centrifugation of the yeast suspension. The glucose control served to prove the activity of the yeast suspension.

# CALCULATIONS

Galactose has a lower reducing power than glucose 1.3 Gm. of C.P. galactose ( $[\alpha]D = + 80.5^\circ$ ) are equivalent to 1.0 Gm. of glucose in the reduction of equivalent amounts of Benedict's or Rudisch's quantitative sugar reagents.

The sugar content is determined before and after fermentation. The non-fermentable fraction is considered to consist of galactose. A correction is made for the galactose which may have disappeared from the mixture as determined by the galactose control. The fermented fraction represents the glucose concentration. Since the sugar content before fermentation was calculated on the basis of the assumption that all the sugar in the test urine was galactose, the figure obtained by subtracting the sugar concentration after fermentation from that before fermentation must be divided by 1.3 to convert this assumed galactose into a glucose value. For example:

	BEFORE FERMENTATION	AFTER FERMENTATION	GRAMS GLUCOSE
Control galactose solution	1.5%	1.4% (0.1%)*	—
100 c.c. test urine	1.9%	1.6% (1.7%) (Corrected)	0.30 (0.20)† (Corrected)

\*Correction for galactose diffusion into yeast suspension

†Fermentable sugar divided by 1.3 to give glucose value  $\frac{0.20}{1.3} = 0.15$  Gm. glucose. (One gram of glucose is equivalent to 1.3 Gm. of galactose in the reduction of Benedict's and Rudisch's solution.)

# RESULTS

In 80 tests made on patients with suspected liver disease with normal and disturbed galactose tolerance, fermentation over a period of twenty-four hours disclosed a disappearance of reducing substance varying between 0.1 and 0.7 per cent, in the majority of instances, 0.3 per cent. Under these circumstances the "fermentable" fraction was found to constitute as much as 30 to 40 per cent of the total reducing substance in the urine. However, in this series corrections were not made for diffusion of galactose into the yeast suspension over a period of twenty-four hours. In clinical laboratories this correction has not customarily been made when glucose-galactose mixtures have been fermented, leading in diabetic urine to erroneous conclusions concerning the amount of "fermented" glucose present.

In 12 instances the disappearance of reducing substances from a galactose solution added to normal urine was compared at the end of one hour and at the end of twenty-four hours of fermentation. At the end of one hour there was no change in 9 of the 12 tests. The amount that disappeared in the 3 remaining tests were 0.10, 0.10, 0.13 per cent, respectively. At the end of twenty-four hours these same tests showed a decrease in reducing substance varying from 0.10 to 0.38 per cent.

TABLE I  
MODIFIED GALACTOSE TOLERANCE TEST IN 10 PERSONS WITH SUSPECTED LIVER DAMAGE

CASE NO.	GALACTOSE URINE CON- TROL PER CENT LOSS	1ST HOUR PER CENT			2ND HOUR PER CENT			3RD HOUR PER CENT			4TH HOUR PER CENT			TOTAL GRAMS			DIAGNOSIS
		B.F.*	A.F.†	NET LOSS	B.F.	A.F.	NET LOSS	B.F.	A.F.	NET LOSS	B.F.	A.F.	NET LOSS	SUGAR GALACTOSE‡		GLUCOSE	
														BAUER	A.F.		
1	0	1.37	1.37	0	5.5	4.7	0.8	2.54	2.3	0.24	0.8	0	6.67	5.93	0.64	Toxic hepatitis	
2	0	1.30	1.22	0.08	0.97	0.87	0.10	0	-	-	0	-	1.36	1.25	0.09	Graves' disease	
3	0	1.76	1.62	0.14	-	-	-	-	-	-	1.17	1.04	4.57	4.20	0.28	Portal cirrhosis	
4A	0.03	3.5	3.2	0.27	2.90	2.64	0.23	1.5	1.38	0.09	0	-	5.46	5.03	0.32	Toxic cirrhosis	
B	0	4.8	4.25	0.55	1.98	1.89	0.09	0.92	0.92	0	0	-	6.75	6.32	0.34		
5	0	1.95	1.3	0.65	3.2	2.26	0.94	2.6	1.5	1.1	1.6	1.05	5.69	3.65	1.58	Cholecholelithin- sis; diabetes mellitus	
6	0	1.3	1.3	0	0.81	0.81	0	0.74	0.74	0	0	-	2.69	2.69	0	Toxic hepatitis (recovery)	
7	0.12	1.58	1.38	0.08	1.44	1.32	0	0	-	-	0	-	4.8	4.73	0.05	Infectious jaundice	
	0	0.37	0.37	0	0.75	0.60	0.15	0	-	-	0	-	2.23	2.05	0.14		
8	0	1.46	1.46	0	1.05	1.05	0	0	-	-	0	-	1.41	1.41	0	Graves' disease	
9	0	1.6	1.6	0	0	-	-	0	-	-	0	-	4.56	4.56	0	Carcinoma of gall- bladder; jaundice	
10	0.03	1.86	1.72	0.11	0	-	-	0	-	-	0	-	2.80	2.64	0.12	Pigment cirrhosis	

\*B.F. = Before fermentation.

\*B.F. = Before fermentation.

†A.F. = After fermentation.

‡B.F.-A.F.

1.3

In 12 tests observations were made on the disappearance of reducing substance from control galactose solutions added to urine at the end of fermentation for one hour only. In this series there was only a single instance of disappearance of reducing substance, in which case 0.12 per cent disappeared.

The percentage decrease of reducing substance at the end of one hour in 24 tests occurred as follows: In 21 instances there was no change; in the 3 remaining instances decreases of 0.10, 0.10, and 0.12 per cent, respectively, were found. In 12 tests observed at the end of twenty-four hours, 6 showed decreases of 0.10 to 0.20 per cent; 2, decreases of 0.20 to 0.30 per cent; and 4, decreases of 0.30 to 0.38 per cent.

In Table I are listed the results in 10 cases of jaundice or disease associated with liver damage. In 4 cases the galactose tolerance was normal, i.e., total excretion was less than 3.0 Gm. In 3 instances the reducing substance found in the urine was found to occur entirely in the form of galactose. Two were in normal tests, the third in a pathologic galactosuria. In 2 tests less than 0.1 Gm. of fermentable substance was present. In the remainder, the amount of glucose present varied between 0.12 Gm. to 0.65 Gm. In a single instance (Case 5) 1.58 Gm. of glucose was present. In this case there was an associated diabetes. The pathologic galactosurias in the series varied between 3.65 Gm. and 6.32 Gm. Excluding the glycosuria associated with diabetes, glycosurias of 0.28, 0.32, 0.34, and 0.64 Gm. were encountered in patients with galactosurias of 4.20, 5.03, 6.32, and 5.93 Gm., respectively. Glycosuria was noted in individual fractions only, and in Case 4 (Test A) in every fraction of urine containing galactose.

#### COMMENT

Experimental studies in galactose tolerance in man and animals have indicated that a reducing substance other than galactose may occur in the urine following the oral and intravenous administration of galactose.<sup>7-13</sup> Under pathologic conditions involving the liver this tendency might be expected to be even more marked. It is difficult to conclude whether the glycosurias associated with galactosuria are primarily the result of the conversion of galactose to glucose or whether the glucose voided in the urine is the result of a displacement of this sugar in the liver following galactose ingestion.

While the amounts of glucose voided in the urine have not been large enough in this small series to alter appreciably the results of the galactose tolerance test, the modified procedure may prove to be of especial diagnostic significance in borderline cases, with values of 3.0 to 4.0 Gm. of galactose.

The galactose control solution is necessary in the accurate estimation by fermentation of sugars in galactose-glucose mixtures. Failure to account for amounts of galactose which diffuse into yeast cells leads to erroneous conclusions favoring the amounts of glucose present in such mixtures. This caution applies especially to the performance of the galactose tolerance test in subjects with diabetes mellitus. The modification introduced here of limiting the period of fermentation to sixty minutes at elevated temperature reduces the rate of diffusion of galactose into yeast cells practically to nil, and at the same time permits the complete fermentation of as high as 2.5 per cent of glucose.

## SUMMARY

A modification of the galactose tolerance test is introduced without interfering with its simple routine clinical use in persons with suspected liver disease.

The modification depends upon the selective fermentation of glucose in galactose-glucose mixtures; under the conditions of the test, galactose being nonfermentable. The possible disappearance of galactose by diffusion into yeast cells is taken into account. The fermented fraction then represents glucose.

Glucose may appear in the urine, associated with normal and pathologic alimentary galactosuria. There is a tendency for increasing amounts of glycosuria to occur with increasing alimentary galactosuria.

The modified technique improves the accuracy of estimation of mixtures of galactose and glucose present in urine in the routine application of the galactose tolerance test in persons with suspected liver damage and with diabetes mellitus.

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## A METHOD FOR THE QUANTITATIVE ESTIMATION OF CHEMICAL IRRITATION\*

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IN MANY biological studies it is frequently desirable to measure quantitatively the irritating properties of chemicals. Heretofore, one has had to depend largely on a personal sensation of pain and on intensities of hyperemia and inflammation produced by irritants, with only one's memory of previous observations upon which to base a comparison. Such is the method described by Munch.<sup>1</sup> In the absence of a more accurate method, information obtained in such a manner may be of considerable value. However, memory is at best an unreliable faculty; and from a scientific point of view, data in which memory of previous experiences plays a significant part are of limited value because of the difficulty of independent repetition and verification. A few investigators have made use of various physical means for measuring the intensity of inflammation, relying on hyperemia or edema to provide an index of the severity of the reaction. While the degree of redness of an inflamed area can be measured with satisfactory accuracy, as described by Oettel,<sup>2</sup> it is not established that redness is a constant and reliable index of the degree of inflammation. Moreover, such measurements usually require elaborate and expensive apparatus. Further, it is only under specialized conditions that redness bears a quantitative relationship to the inflammatory process with which it is associated. The use of edema as a criterion is likewise subject to many objections, among which is the very great variation in results obtained when the same chemical is applied to the same type of tissue. Thus, Haag<sup>3</sup> found very great variations in the amounts of edema produced by irritants instilled into the conjunctival sacs of rabbits' eyes. Also, the same solution of an irritant frequently produced no visible edema in the eyes of one rabbit, but marked edema in those of another.

Ebbecke,<sup>4</sup> Hirschfelder,<sup>5</sup> and Menkin<sup>6</sup> observed that trypan blue, when injected intravenously, becomes fixed at all sites of inflammation. Regardless of the exact mechanism of this fixation, if the intensity of the blue color resulting from the accumulation of dye at the site of inflammation is proportional to the intensity of inflammation, a potential method is at hand by which the relative strengths of chemical irritants may be measured quantitatively. Tainter, Thronsdon, and Lehman<sup>7</sup> applied this principle in a very limited number of observations, but apparently did not extend their observations to include more than a single chemical, sodium bisulfite.

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In these experiments a variety of chemicals was used, both as solutions of a single substance and in mixtures. Up to the present time well over fifteen hundred rabbits have been used for these observations. The results from a few representative chemicals are reported here.

#### EXPERIMENTAL METHODS

Adult white rabbits of both sexes were used as test animals throughout these experiments. Gray or brown rabbits were found unsatisfactory because the skin pigments partially obscured the dye. Solutions of irritants, the irritating properties of which were to be studied, were injected subcutaneously into the dorsal surfaces of the ears, usually at three sites on each ear. Intracutaneous injections into the skin of the abdomen were tried, but were found less satisfactory for two reasons: first, the skin of the abdomen seemed to be less sensitive to the action of irritants; and second, slight trauma to the skin caused by removing the fur either by shaving or by using barium sulfide as a depilatory, was frequently sufficient to cause the accumulation of so much dye in the skin as to interfere with the evaluation of the intensity of spots at the sites of injection of the solutions being tested. The intravenous injection of the dye was made into one of the superficial veins of the abdomen in most instances, but occasionally into one of the marginal veins of the ear. It was considered advisable to avoid injections into ear veins as far as possible, lest manipulation and trauma interfere in some way with the uniform distribution of dye in the two ears. The various irritant solutions were injected in  $\frac{1}{20}$  c.c. quantities. The amount of trypan blue used was 1 c.c. per kilogram of 1 per cent solution in 0.85 per cent sodium chloride. This quantity of dye was much less than that used by Menken, but proved to be adequate. Moreover, larger quantities of dye frequently resulted in such intense coloration at the sites of inflammation that comparison with a standard color scale became difficult or even impossible. The standard color scale was prepared by placing drops of solutions of various concentrations of the dye on a white background, for which blotting paper was found satisfactory. In the scale used the concentration of dye varied from a maximum of 0.05 per cent to a minimum of 0.0029 per cent. There were eight different concentrations in the scale, and each was two-thirds as high as the one above it. Thus, No. 8 was 0.05 per cent, No. 7 was 0.033 per cent, No. 6 was 0.022 per cent, and so on.

It was found that solutions of different concentrations of an irritant produced blue spots which, within a limited range, were proportional in intensity to the concentrations of the solutions. However, the maximum range of color between a slight, though definite, blueness, and an intensity beyond which no further increase could be noted, corresponded to only a relatively limited range of concentration of the irritant. Also, it was found that any given solution of an irritant produced spots which varied in intensity over a range of two or three points on the standard scale. Therefore, in order to have the method yield quantitative results it was necessary to assign to each degree of intensity on the color scale an arbitrary numerical value. It was found experimentally that if these arbitrary numerical values were propor-

tional to the concentration of dye in the standard scale, the degree of irritation, as indicated by this numerical value, was approximately proportional to the concentration of the irritant solution. Thus, doubling the concentration of the irritant solution gave values which were approximately doubled. Frequently traces of dye were observed at the sites of injection which were less than the lowest intensity on the color scale. These were designated as "traces," and assigned a value in keeping with the remainder of the scale. The arbitrary values selected are as follows: Trace, 0.66; No. 1, 1.0; No. 2, 1.50; No. 3, 2.25; No. 4, 3.37; No. 5, 5.06, etc. Concentrations of irritant solutions were selected which gave values rarely as high as No. 4 (3.37). With such concentrations the values ordinarily varied between 0.66 and 2.25, with an average for several animals of 0.9 to 1.5.

Observations extending over a period of about eighteen months indicate that certain climatic conditions, particularly temperature, greatly influence the intensity of the blue spot. This is to be expected when it is considered that circulation through vessels in the rabbit's ear apparently changes greatly with changes in temperature. This fluctuation between peripheral vasoconstriction and vasodilatation was observed to produce results varying by as much as 100 per cent. Presumably air-conditioned animal rooms and laboratories would greatly reduce such fluctuations, but in the absence of such uniform conditions of temperature and humidity it is necessary to compensate in another manner. The procedure found to give satisfactory results is to inject a standard irritant solution into one ear of the animal and the unknown solution into the other. Then, by dividing the average value for the unknown solution by that for the standard solution a quotient is obtained which represents the ratio, Unknown/Standard. The standard irritant solution routinely used contains 0.1 N acetic acid, 0.1 N sodium acetate, and 0.1 per cent benzoic acid. This mixture has a pH of 4.68; it is stable and easily reproducible. Immediately before use it is diluted to four volumes, so as to contain 0.025 N acid and salt, and 0.025 per cent benzoic acid. Triply distilled water was used in the preparation of all solutions.

It was found that various nonirritant substances, such as 0.85 per cent sodium chloride solution and liquid petrolatum, caused the accumulation of traces of dye at the sites of injection if the dye was administered immediately after the injections were made. However, if a period of about forty minutes was allowed to elapse between the injection of irritant and the injection of dye, there was no significant accumulation of dye following the injection of 0.85 per cent sodium chloride solution. It was, therefore, uniformly the practice to wait forty minutes after injections of irritants before giving the dye. Liquid petrolatum caused the accumulation of dye even if the usual period of forty minutes was extended to two or three hours; for this reason, it was not used as a solvent in any of the experiments reported here. Occasionally, though rarely, sufficient trauma was produced by the injection to give a faintly positive reaction, even though a nonirritant solution was used. This was minimized by the use of hypodermic needles of not larger than 27 gauge. Occasionally small blood vessels were damaged by the injection, so as to result in a small hemorrhagic spot which contained little or no dye. Such spots

were omitted in the final observations. Injections of distilled water uniformly produced a definite accumulation of dye, so all solutions were made isotonic. Following the injection of irritant solutions, the accumulation of dye was usually evident within ten or fifteen minutes, and reached maximum intensity within forty-five minutes, at which time final comparison with the standard color scale was made. The persistence of the blue spots was more or less proportional to the degree of inflammation. Spots from barely irritating concentrations were usually gone within twenty-four hours, but if the concentration of the irritant solution was sufficient to produce spots corresponding to 7 or 8 on the standard scale, they usually persisted for several days, or even weeks. No attempt was made to measure persistence of spots with greater accuracy because of the interference from dye which always accumulated in subcutaneous tissues after eighteen to twenty-four hours.

For approximate results 3 or 4 animals were usually sufficient, and the standard irritant solution was omitted. For more accurate results 16 animals were found to suffice when the standard irritant solution was used in one ear of each animal. Analyses by statistical methods of results so obtained indicate that differences of the order of 15 to 20 per cent can be detected with a degree of certainty of 99 per cent, which compares favorably with many other accepted methods of bio-assay.

Due to the persistence of the dye in tissues of animals they cannot be used a second time within less than about six weeks after the first experience.

TABLE I

1 SUBSTANCE	2 MOLAR CONC.	3 UNKNOWN IRRITATION VALUE	4 STANDARD IRRITATION VALUE	5 UNKNOWN STANDARD	6 COR- RECTED MOLAR CONC.	7 CORRECTED UNKNOWN STANDARD
HCl	0.001	$1.07 \pm 0.056$	$1.39 \pm 0.060$	$0.77 \pm 0.050$	0.0013	$0.82 \pm 0.067$
CH <sub>3</sub> COOH	0.003	$0.91 \pm 0.050$	$1.49 \pm 0.060$	$0.61 \pm 0.040$	0.0049	$0.81 \pm 0.029$
NH <sub>4</sub> OH	0.02	$0.91 \pm 0.042$	$1.30 \pm 0.042$	$0.70 \pm 0.039$	0.0286	$0.81 \pm 0.047$
HCHO	0.04	$0.79 \pm 0.036$	$1.10 \pm 0.043$	$0.72 \pm 0.045$	0.0556	$0.89 \pm 0.044$
NaHSO <sub>3</sub>	0.015	$0.95 \pm 0.041$	$1.38 \pm 0.043$	$0.69 \pm 0.054$	0.022	$0.88 \pm 0.046$

## RESULTS

Substances studied according to the method described herein include acids, bases, salts, glycols, and formaldehyde. Results are shown in Table I. In this table are included molar concentrations of irritant solutions used (2); average irritation values of solutions, as obtained from observations on 16 animals for each (3); average irritation values of the standard irritant solution obtained simultaneously (4); the ratio unknown/standard (5); predicted molar concentrations of irritant solutions which should give a ratio unknown/standard of 1 (6); and the ratio unknown/standard actually obtained from these predicted molar concentrations (7). Figures in column 6 were obtained by dividing molar concentration actually used (2) by unknown/standard (5). Therefore, corrected molar concentrations (6) represent concentrations which should be equally irritating among themselves and also with the standard solution. In column 7 are shown figures obtained from the corrected molar concentrations.

These figures uniformly approach those for the standard more closely. With formaldehyde the error is well within the limits of the method—15 to 20 per cent—but with acids and ammonia the agreement is not as close as might be desired. A third experiment, the results of which are not shown in the table, was performed with ammonia. A second corrected molar concentration was calculated just as was done for the first, except that the calculation was based on corrected unknown/standard of 0.81 instead of on unknown/standard of 0.70. The solution prepared on the basis of this calculation gave a ratio for unknown/standard of 1.00, which is in perfect agreement with the theoretical value.

Two possible explanations may be offered for the lack of better agreement between actual and predicted values for the acids and ammonia: (1) intensity of the blue color is not exactly proportional to concentration of the irritant solution; and (2) in spite of the fact that triply distilled water was used throughout these experiments in preparing solutions, sufficient buffering material may have been present to alter the hydrogen-ion concentration to a significant extent. Frequent pH measurements with a glass electrode indicated that it is difficult to prepare such dilute solutions of electrolytes and not have the pH altered slightly by traces of buffering substances which are so nearly impossible to remove. The second explanation appears even more probable in view of the fact that when the standard solution at a concentration of 0.015 N acid and salt was compared with the same standard at the usual concentration of 0.025 N, the ratio was 0.66. When a concentration of 0.020 N was compared in the same manner, the ratio was 0.81. These two ratios are to be compared with theoretical ratios of 0.60 and 0.80, assuming a direct proportion between concentration and intensity of blueness. It is obvious that a buffered solution, such as the standard, would be much more stable in the presence of traces of buffering material than dilute solutions of acids and ammonia, such as those used.

Sodium hydroxide appeared to be two or three times as irritating as ammonium hydroxide, although reliable figures were difficult to obtain. At concentrations less than 0.01 N there appeared to be little accumulation of dye; at higher concentrations the usual effect was production of a blue ring about one-half inch in diameter, having a clear area in the middle. It appeared as though there was tissue destruction at the site of injection. Due to this atypical appearance strong bases were not investigated further.

Acetates and formates of nicotine and ammonia showed practically no irritating properties in concentrations less than isotonic, and are, therefore, not included in the table. Sodium bisulfite forms a relatively strongly acidic solution, and acts similarly to other buffered solutions, such as the standard. The results obtained with sodium bisulfite confirm those reported by Tainter, Thronson, and Lehman. The glycols (propylene glycol, diethylene glycol, and glycerin) produced no significant degree of inflammation at concentrations less than 2 or 3 moles per liter. Since isotonic concentrations of nonelectrolytes are usually considered to be about 0.29 molar, one is justified in con-

cluding that irritation from such concentrations of glycols as the above was the result of hypertonicity rather than of inherent chemical properties of these substances.

Numerous tests on the relative irritating properties of solutions of cigarette smoke prepared from cigarettes containing glycerin as the hygroscopic agent and those containing diethylene glycol failed to show a significant difference between the two types. Smoke solutions were prepared according to the gravity collection method described by Bradford, Harlan, and Hanmer.<sup>8</sup> All experiments were conducted in such a manner that the operator was not aware of the identity of the solutions being tested.

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### ESTIMATION OF THIOCYANATES IN THE BLOOD WITH THE USE OF PERMANENT STANDARDS\*

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RECENT reports indicate that thiocyanate therapy may be a useful remedy for certain types of hypertension, provided the treatment is properly controlled by means of blood thiocyanate determinations.<sup>1-3</sup> For clinical uses the method for blood thiocyanate determination to be described presents one or more of the following advantages over previously described methods: (1) No expensive equipment is necessary. (2) Repeated preparation of standard solutions is eliminated. (3) A determination can be made in a few minutes. (4) A determination can be made on 1 c.c. or even 0.25 c.c. of serum.

#### MATERIALS

1. Twenty per cent trichloroacetic acid solution.
2. Ferric nitrate reagent: Dissolve 5 Gm. of crystallized ferric nitrate in 50 c.c. of distilled water. Add 2.5 c.c. of concentrated nitric acid and make up to 100 c.c. with distilled water.

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3. Standards: Dissolve 0.0567 Gm. of potassium dichromate and 3.83 Gm. of cobalt chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) in 100 c.c. of distilled water. At times it may be more satisfactory to use larger quantities, that is, 0.567 Gm. of potassium dichromate and 38.3 Gm. of cobalt chloride in 1,000 c.c. of distilled water. The standard solution thus produced corresponds to 10 mg. of thiocyanate per 100 c.c. of serum. Other standards are made by proportional dilution of this standard solution; that is, the 5 mg. standard is obtained by mixing 5 c.c. of the 10 mg. standard and 5 c.c. of distilled water; the 4 mg. standard, by mixing 4 c.c. of the 10 mg. standard and 6 c.c. of distilled water, etc. Each standard is sealed in a comparator tube  $\frac{7}{16}$  inch in diameter. If a comparator block with two rows is used, the standards absolutely essential are the 1, 2, 3, 4, 5, and 10 mg. standards. Since, however, the important therapeutic range is from 5 to 10 mg. per cent, it is possibly of some advantage to have all standards from 1 to 10. A blank of distilled water is essential.

4. Three or 4 comparator tubes calibrated for 1 and 2 c.c. Needless to say, the comparator tubes must be matched.

5. A comparator block with two rows. If only one row of the comparator block is used, the row next to the frosted glass is used. If both rows are used, the unknown solution must be balanced with the tube of distilled water.

6. A dropper which delivers 0.2 c.c. (for use with the ferric nitrate reagent).

7. A dropper which delivers 0.5 c.c. (for use with the trichloroacetic acid solution).

8. One or 2 droppers calibrated to deliver 0.25, 0.5, 0.75, and 1 c.c. (for measuring serum).

9. One or 2 small funnels.

10. Filter paper to fit the funnels.

#### PROCEDURE

In one of the comparator tubes put water to the 2 c.c. mark. Add 0.5 c.c. of trichloroacetic acid solution and 1 c.c. of serum and mix well. Filter into another comparator tube until 1 c.c. of clear filtrate is obtained. To this filtrate add 0.2 c.c. of ferric nitrate reagent. The color that forms is matched in the comparator with the standards. The reading is in milligrams per 100 c.c. of serum.

If 1 c.c. of serum is not obtainable, or if the reading with 1 c.c. of serum is over 19 mg. per cent, 0.5 or 0.25 c.c. of serum may be used and 0.5 or 0.75 c.c. of water, respectively, is added. The result must then, of course, be multiplied by 2 or 4 as the case may be.

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## METABOLISM OF ALLANTOIN\*

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### APPEARANCE IN THE URINE ON FEEDING AND RELATED EXPERIMENTS

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ALLANTOIN is the chief end product of purine metabolism with most mammals. It is due to the uricolytic enzyme, uricase, which is found in the liver of these animals and converts uric acid into allantoin. According to Oikawa,<sup>1</sup> uricase, which he prepared from rabbit liver, acts best at 48° C. and is destroyed at 70° C. It is most active in an atmosphere of oxygen at a pH of 7.1 to 7.2, less active in an atmosphere of nitrogen, and practically inactive in carbon dioxide, carbon monoxide, and hydrogen atmospheres, respectively. Its activity is not effected by autolysis.



## PRELIMINARY EXPERIMENTS

Prior to the actual feeding experiments the effect of the various gastrointestinal enzymes at their *in vivo* acidity or alkalinity, as the case may be, on standard solutions of allantoin was determined. Artificial gastric juice was prepared by digesting 10 Gm. of minced pigs' stomach mucosa with 50 c.c. of 0.4 per cent hydrochloric acid solution for twenty-four hours at 37° C., filtered and dialyzed to remove peptones and peptides. Intestinal enzymes were extracted from minced pigs' pancreas, using 1 per cent sodium bicarbonate in the proportion of 1 Gm. of organ to 3 c.c. of bicarbonate.

Standard allantoin solutions of varied concentrations were incubated in these enzymic extracts for a period of four hours at 37° C., and subsequently analyzed for allantoin. The results appear in Protocol I and obviously indicate no breaking of the allantoin.

## PROTOCOL I

## EFFECT OF GASTROINTESTINAL ENZYMES ON ALLANTOIN

ENZYMIC EXTRACT	ALLANTOIN ADDED MG. PER C.C.	ALLANTOIN RECOVERED MG. PER C.C.
Gastric	1.0	1.0
	1.5	1.5
	2.0	2.0
	2.5	2.5
Intestinal	1.0	1.0
	1.5	1.5
	2.0	2.0
	2.5	2.5

Rabbits were then fed definite amounts of allantoin. This was done by inserting a tube through the pharynx into the stomach, attaching a 10 c.c. syringe to the exposed end, placing the allantoin in the barrel of the syringe, and washing it through the tube with small amounts of water, with the assistance of slight pressure on the plunger. The animals were placed in metabolic cages and successive twenty-four-hour specimens of urine and feces were collected. The specimens of feces were triturated, extracted with water, and filtered. The filtrates were analyzed in conjunction with the urines for allantoin content, utilizing the method described by Larson.<sup>4</sup> No allantoin could be detected in the feces extracts in contrast to the urine analysis which gave tremendous recoveries of allantoin. The analytical data of these determinations are tabulated in Protocol II.

## PROTOCOL II

## RECOVERY OF ALLANTOIN AFTER INGESTION OF TARED AMOUNTS

RABBIT NO.	ALLANTOIN INGESTED	ALLANTOIN RECOVERED IN FECES	ALLANTOIN RECOVERED IN URINE	PER CENT RECOVERY
456	0.56 Gm.	None	1.40 Gm.	250
457	0.54 Gm.	None	1.76 Gm.	326
458	0.57 Gm.	None	1.75 Gm.	307
459	0.55 Gm.	None	1.84 Gm.	334

It was soon discovered, however, that allantoin ingestion greatly increased the excretion of creatine,<sup>5</sup> which in turn reduces the alkaline copper solution

utilized in this method for determining allantoin. Since creatine is not removed in the preliminary preparation of the specimen, it was the cause for the abnormally high results.

Protocol III shows this effect in tabulated form. In a large number of rabbit urines analyzed for both creatine and allantoin, allantoin is manifested chiefly in those urines containing creatine, and the concentration varies directly with the quantity of creatine present.

### PROTOCOL III

#### EFFECT OF CREATINE ON ALLANTOIN DETERMINATION (LARSON)

URINE	CREATINE	ALLANTOIN
A1	3.2	5.0
A2	1.3	3.0
A4	0	0
A7	0	3.6
A8	9.3	7.4
B3	0	0
B5	4.7	6.7
B6	5.45	7.35
B7	0.5	0
B8	2.0	3.0

It was, therefore, necessary to develop a more specific method for the allantoin determination. The method finally adapted entirely eliminates the creatine present. It prepares the specimen according to the usual procedure, then utilizes the quantitative precipitating action of mercury salts on allantoin, producing mercuric allantoinate.

### METHOD

The specimen is treated with an excess of 25 per cent phosphotungstic acid, followed by an excess of basic lead acetate and 5 per cent sulfuric acid solution. This removes most of the interfering substances. The entire procedure is carried out in the same pyrex tube. After the addition of each reagent the tube is rotated to insure thorough mixing and then centrifuged until clear. Two cubic centimeters of the supernatant liquid are transferred to another pyrex tube, neutralized to litmus, and the allantoin isolated by precipitating it with mercuric acetate-sodium acetate reagent. After standing one hour the mixture is centrifuged and the supernatant fluid is discarded. The precipitated mercuric allantoinate is then washed several times with water, rubbed thoroughly each time with the aid of a rubber policeman to insure penetration of the water, and centrifuged. All the wash water is discarded. The precipitate is finally dissolved in acetic acid and the nitrogen content is determined by micro-Kjeldahl analysis.

Reagents required: Phospho-24-tungstic acid.<sup>6</sup> This substance was prepared in our laboratories because the commercial grades are too impure for satisfactory results.

Basic lead acetate.<sup>7</sup>

Sulfuric acid solution, 5 per cent (by weight).

Sodium hydroxide, 5 per cent solution.

Mercuric acetate-sodium acetate reagent: Dissolve 1 Gm. of mercuric acetate and 10 Gm. of sodium acetate in water and make up to 100 c.c.

Glacial acetic acid, 20 per cent solution.

Litmus solution indicator.

Concentrated sulfuric acid.

Copper sulfate, 5 per cent solution.

Nessler's solution.<sup>8</sup>

#### PROCEDURE

Three and five-tenths grams of phosphotungstic acid are dissolved in water and diluted to 10 c.c. Two cubic centimeters of this solution are transferred to a pyrex tube and 2 c.c. of the specimen to be analyzed are added. Mix thoroughly and centrifuge. To the same tube add 2 c.c. of the basic lead acetate reagent, mix and centrifuge again. Finally, add 2 c.c. of the 5 per cent sulfuric acid solution and centrifuge until clear. Transfer 2 c.c. of the clear supernatant liquid to another pyrex tube and neutralize to litmus, using 5 per cent sodium hydroxide. After neutralization add 1 c.c. of the mercuric acetate-sodium acetate reagent, mix thoroughly, and set aside for one hour. Centrifuge and decant the supernatant fluid. Wash the precipitate several times with water, rubbing the precipitate each time with a rubber policeman attached to the end of a stirring rod, centrifuging and decanting. After the final decantation add 1 c.c. of 20 per cent glacial acetic acid to the precipitate and rub up until solution is affected. Then neutralize to litmus with a 5 per cent sodium hydroxide solution and make the micro-Kjeldahl<sup>9</sup> nitrogen determination as follows: Transfer the prepared sample to a Folin digestion tube which is conveniently graduated at the 35 and 50 c.c. marks. To this add 0.5 c.c. of concentrated sulfuric acid, 2 drops of a 5 per cent solution of copper sulfate, and several glass beads to prevent bumping. Then boil the mixture vigorously until the moisture is driven off and sulfur dioxide fumes fill the tube. Continue boiling the contents slowly until the fumes are driven off. The mixture should now appear pale blue green or yellowish in color. Allow the tube and contents to cool, dilute to the 35 c.c. mark with water, and add Nessler's solution to the 50 c.c. mark. Mix thoroughly and compare with an allantoin standard, standardized to contain 0.1 mg. of nitrogen per c.c., which has been digested in the same manner as the unknown. The unknown and standard should be nesslerized as nearly simultaneously as possible.

#### CALCULATIONS

$$X = \frac{(\text{Reading of standard}) (\text{Concentration of standard}) (\text{Dilution factor})}{(\text{Reading of unknown}) (\% \text{ nitrogen per mole of allantoin})}$$

The value for X will indicate the milligrams of allantoin per cubic centimeter of unknown.

#### REMARKS

The foregoing procedure was designed for use with urines, but has been used successfully with extracts of other material. The quantities of reagents cited are sufficient for the average normal urine specimens. However, should the material in question contain unusually high concentrations of proteins and their metabolic products, either the analysis should be diluted before performing or the proper balance of reagents should be determined. The former is obviously the better procedure.

The phosphotungstic acid precipitates the proteins while the addition of basic lead acetate removes the excess phosphotungstic acid and some other interfering substance. The subsequent addition of sulfuric acid precipitates the excess lead. The formation of a precipitate on neutralizing with sodium hydroxide indicates that the lead had not entirely been removed. On addition of acetic acid the precipitate may not entirely dissolve. This insoluble material is not mercuric allantoinate, but, at least a portion, is uric acid; therefore, remove by centrifuging and utilize the clear supernatant liquid only. A standard solution of allantoin is used as the comparison standard due to the difference in hue of the color developed on nesslerization.

## EXPERIMENTAL

Rabbits were placed in metabolic cages and several twenty-four-hour specimens of urine were collected and analyzed to determine the daily normal excretion of allantoin. These animals were then fed weighed amounts of allantoin in the same manner as previously described, and several additional twenty-four-hour specimens of urine were collected and analyzed for allantoin. The tabulated results of these analyses appear in Protocol IV.

## PROTOCOL IV

## RECOVERY OF ALLANTOIN FED TO RABBITS

RABBIT NO.	ALLANTOIN FED	ALLANTOIN RECOVERED	PER CENT RECOVERY
	mg.	mg.	
775	536	628	117.16
776	500	486	97.20
777	550	333	60.54
778	550	1039	188.90
779	530	983	185.47
780	562	1048	186.48
781	538	477	88.66
782	530	979	184.70
783	590	884	149.83
784	510	1331	260.98
785	500	1133	226.60

With few exceptions the results indicate recoveries ranging from 100 per cent to 200 per cent, with one instance of 260 per cent recovery. The excess over that normally present and that ingested seems to indicate that possibly some organ retains the substance which, during periods of prolonged fasting, can be utilized in the resynthesis of cellular products, and the forced feeding of amounts in the concentrations given stimulates that organ to the point of causing this allantoin to be excreted. To obtain more information on this theory, analyses were made on aqueous extracts of macerated kidneys and livers of both normal and allantoin fed rabbits. The results for the liver analysis are recorded in Protocol V.

These results appear to indicate the validity of the afore-mentioned theory and point to the liver as the organ retaining a concentration, for in normal liver a concentration of 0.44 mg. to 0.60 mg. of allantoin per gram of organ is found in comparison to 0.09 mg. to 0.35 mg. of allantoin per gram of organ in animals that were fed 0.5 Gm. of the substance eight hours previously. On the other hand, normal kidneys show the presence of allantoin to the extent of 0.1

## PROTOCOL V

## EFFECT OF ALLANTOIN INGESTION ON ALLANTOIN CONTENT OF LIVER

RABBIT NO.	MG. ALLANTOIN PER GM. OF ORGAN	
	NO ALLANTOIN FED	INGESTED 8 HOURS PREVIOUSLY
991	0.44	
992	0.51	
993	0.60	
994	0.49	
995		0.33
996		0.13
997		0.35
998		0.09
Average	0.51	0.225

per gram of organ, whereas the animals fed allantoin show a content of 1.17 mg. per gram of organ.

*Effect of Fasting on Allantoin Content of Liver.*—Three groups of two rabbits each were placed on a starvation diet to determine the effect of inanition on the allantoin content of the liver. One group was sacrificed at the end of three days, another in five days, and the third seven days after inanition.

The results represent the average values for the analysis made on the pooled material of each group. The livers and kidneys were macerated and extracted with twice their weight of water at 40° C. After forty-eight hours' extraction the material was filtered and the filtrate was analyzed for allantoin, utilizing the procedure already outlined.

The analysis of the urines excreted during this period showed a gradual but definite increase of allantoin excretion, paralleling the analytical results for allantoin in kidneys which increased with each successive inanition period.

Results of the liver and kidney analysis appear in Protocol VI.

## PROTOCOL VI

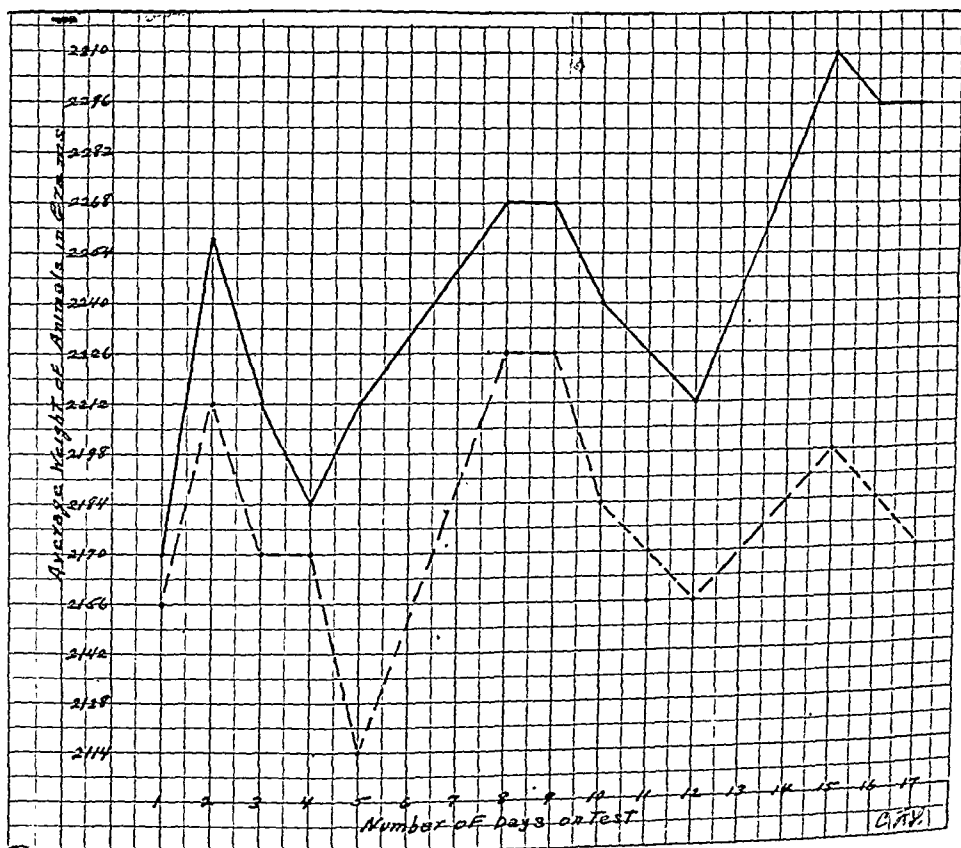
## EFFECT OF FASTING ON ALLANTOIN CONTENT OF LIVER AND KIDNEY

PERIOD OF FAST	MG. ALLANTOIN PER GM. OF ORGAN	
	IN LIVER	IN KIDNEY
3 days	0.61	0.21
5 days	0.32	0.35
7 days	1.0	1.16

The drop in the allantoin content of liver between the first and second inanition periods would tend to support the theory that allantoin can be resynthesized into cellular products during time of fasting. On the other hand, the results obtained after seven days of inanition appear to indicate a tremendous increase of uricolytic enzyme stimulation. According to Oikawa,<sup>10</sup> who reported on the effect of inanition upon the uricolytic activity of the liver extract, during starvation experiments for twenty-four hours with the rabbit, no effect on uricolytic activity was noted in the first week, but some increase was obvious in a longer period of time.

*Effect of Allantoin Ingestion on Body Weight.*—The tremendous increase of creatine excretion prompted experiments to determine whether this creatine represented cellular loss and probably led to eventual depletion of cellular creatine. This was done by feeding various amounts of allantoin to rabbits

daily and observing changes occurring in body weight. Graphs 1 and 2 show the effect of ingested allantoin on body weight of rabbits. Each curve represents the average weight of two rabbits. In Graph 1 the test animals were fed 100 mg. per kilogram body weight per day. As shown by this curve the maximum and minimum weight fluctuation over a period of sixteen days approximates a straight line, indicating a remarkably constant body weight. However, there is definite inhibition of weight increase in these animals.

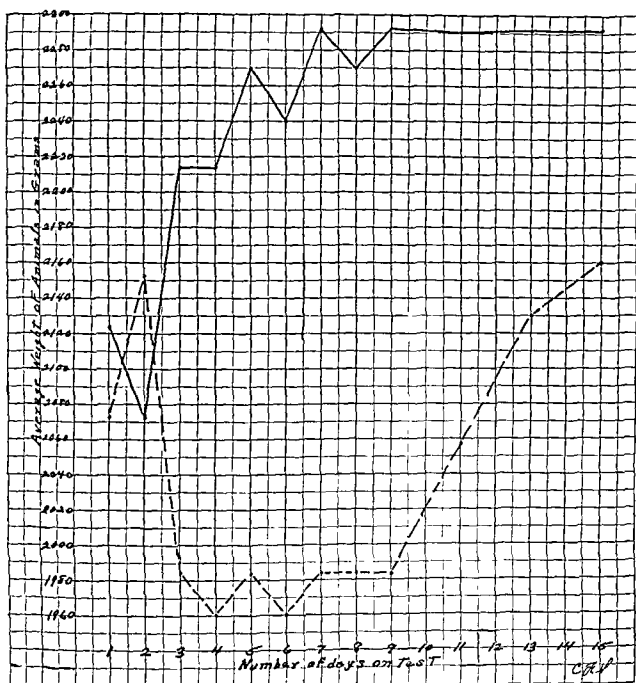


Graph 1.————— Control animals.  
 - - - - - Animals fed 100 mg. per kg. body weight per day.

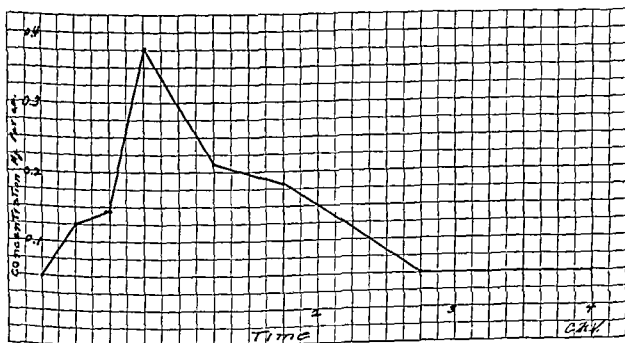
Graph 2 shows the effect of feeding 250 mg. per kilogram body weight per day. Here, too, the curves represent the average weight of two rabbits. The curve representing the test animals shows a decided decrease in weight throughout the test period in contrast to the curve representing the control animals. However, recovery of lost body weight during the test period is rapidly replaced on removal of the allantoin from the diet, as is shown by that portion of the curve lying between the ninth and thirteenth day period.

The feeding of amounts less than 100 mg. per kilogram body weight had no effect on normal body weight increase.

After completion of the work reported here, there appeared the publication of Beard and Pizzolato<sup>11</sup> on the effect of parenteral injection of purines, methylated purines, and various drugs upon creatine-creatinine metabolism in the



Graph 2.————— Control animals.  
 - - - - - Animals fed 500 mg. per kg. allantoin per day.



Graph 3.—Maximum concentration of allantoin in circulation on intravenous injections.

muscles of the rat. They reported that adenine, xanthine, hypoxanthine, uric acid, and allantoin gave increases in muscle creatine as well as large increases in creatine excretion, showing that the urinary creatine did not originate from that in the muscles. They concluded that these substances stimulated creatine formation and excretion of the body in a direct proportion to the number of methyl groups, up to 3, present in the supplement injected.

*Maximum Concentration of Allantoin in Circulation on Intravenous Injections.*—Rabbits were given intravenous injections of allantoin to determine the interval in which the maximum concentration of allantoin appears in the circulation, the amount recovered at that time, and the time required for the injected allantoin to disappear entirely from the blood stream. Forty milligrams of allantoin were injected into each animal and bleedings were made (heart puncture) at fifteen-minute intervals. As shown in Graph 3, forty-five minutes after the injection the maximum concentration of allantoin was manifest. At this point 38.54 mg., or 96.36 per cent, of allantoin were recovered. At the end of two hours and forty-five minutes the blood concentration had dropped to normal.

#### SUMMARY

1. The method described by Larson<sup>4</sup> for the determination of allantoin in urine cannot be used on urines containing creatine. Due to the fact that allantoin injection causes the excretion of large amounts of creatine, it was necessary to develop a procedure which eliminated the creatine entirely. This was done by precipitating the allantoin as the mercuric allantoinate.

2. Allantoin ingested by rabbits is completely absorbed and subsequently excreted by the kidneys. No allantoin could be detected in the intestinal excreta.

3. Allantoin recovery experiments after ingestion of tared amounts by rabbits, indicate quite an excess over that ingested and normally present. This excess was traced, partially at least, to the liver. This organ loses its normal concentration of allantoin when that substance is fed. Experiments on the explanation of this phenomenon are in progress.

4. Effect on body weight of animals fed allantoin daily to determine the probable origin of the increased creatine output, showed a decided loss in weight when amounts above 250 mg. per kilogram of body weight were fed. This would seem to indicate that the excess creatine was derived from cellular creatine.

5. Allantoin injected intravenously reaches its maximum concentration forty-five minutes after injection. At this point 96.36 per cent can be recovered. At the end of two hours and forty-five minutes the blood concentration of allantoin has dropped to normal.

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## TECHNIQUES FOR THE PREPARATION AND CARE OF PANCREATIC FISTULAS IN DOGS\*

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**D**URING the past two years we have prepared and cared for over 250 pancreatic fistula dogs. This experience has enabled us to establish fistulas suitable for *extended* observations. It is believed that an account of the techniques for the preparation and care of these fistulas, which have proved most satisfactory in this laboratory, would be of assistance to those attempting such preparations for the first time.

We have prepared fistulas according to the methods of Babkin,<sup>1</sup> Dragstedt,<sup>2</sup> Elman and McCaughan,<sup>3</sup> and Inlow.<sup>4</sup> No attempt will be made to review these methods; the reader is referred to Babkin,<sup>5</sup> and Boldyreff.<sup>6</sup> The most satisfactory fistulas have proved to be those of Dragstedt and Inlow. The operations and the postoperative animal care, as modified by us, will be described in detail sufficient to facilitate such preparation. All operations are performed with rigorous asepsis and full surgical anesthesia.

### THE DRAGSTEDT FISTULA

In the Dragstedt fistula the pancreatic juice is conducted into a small duodenal pouch through the *undisturbed* major pancreatic duct; a cannula in this pouch conducts the secretion to a football bladder suspended beneath the dog's abdomen. (In Dragstedt's published method the duodenal pouch received *all* the pancreatic ducts. Fistulas of the major pancreatic duct *only* have been used in his laboratory for years.<sup>7</sup>) The cannula (Fig. 1) is made of "18-8" stainless steel and satisfactorily replaces the gold plated one of Dragstedt.

**Operation.**—An 18 to 20 kg. female dog is anesthetized with ether following preoperative morphine and atropine. The abdomen is opened by a 10 cm. incision, starting just below the xiphoid and ending 3 cm. to the right of the midline. The duodenum is grasped with moist sponges and delivered into the wound. The antimesenteric duodenal border is pulled to the right (operator

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on right side of animal), bringing the left side of the gut uppermost. The major pancreatic duct joins the duodenum 3 to 4 cm. rostral to the most caudal application of the pancreas to the duodenum. The common bile duct, which enters the bowel rostral to the major pancreatic duct, is located at its point of entrance into the bowel.

The aseptic end-to-end anastomosis, which has proved most satisfactory in this laboratory, has been developed by Martzloff and Burget<sup>s</sup> (1931). Long Carmalt artery forceps are ground on one side to reduce the blade width to 3 mm.; this facilitates the inversion of a *small* diaphragm.

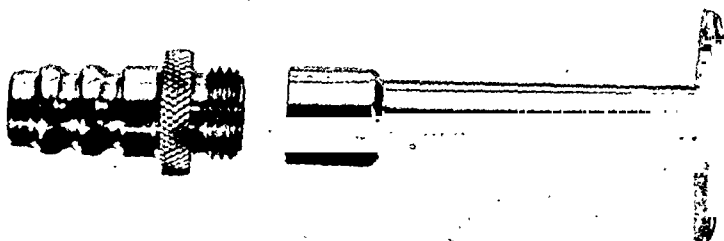


Fig. 1.—Photograph of a 2 inch "18-8" stainless steel cannula with a "screw tip."

Fifteen centimeters of bowel below the common bile duct are packed off outside the abdomen to reduce peritoneal soilage. Midway between the common bile duct and the major pancreatic duct, Martzloff forceps are placed across the bowel. The "ground" side of the forceps is faced rostrad (handle away from the operator). Another forceps (handle toward operator and "ground" side caudad) is placed across the bowel just below and touching the upper forceps. Both forceps are closed as completely as possible. About 8 cm. caudad on the bowel the above procedure is duplicated, except that the distal forceps is set with the "ground" surface caudad (handle away from the operator). The bowel between the upper and lower pairs of forceps is severed flush with the forcep's blades; the cut surfaces are treated with 95 per cent phenol followed by dilute alcohol.

A 1 cm. incision is made midway between the forceps on the antimesenteric border of the duodenal pouch segment. The caudal forceps is removed and the "conical tip" of the cannula (see Fig. 2) is introduced into the pouch lumen and brought out through the antimesenteric wound. The caudal end of the pouch is clamped shut with Martzloff forceps, excess tissue is removed, and the cut ends of the bowel are recauterized with phenol. Both ends of the pouch are closed by oversewing the forceps (Parker-Kerr technique<sup>3</sup>), using No. 1 silk suture on a straight intestinal needle. A purse-string suture is placed in the pouch about the cannula; the wound edges are invaginated and the suture is tied just tightly enough to prevent pericannular leakage.

End-to-end anastomosis of the severed ends of the proximal and distal duodenum is now begun. The Martzloff forceps are turned by the assistant, so

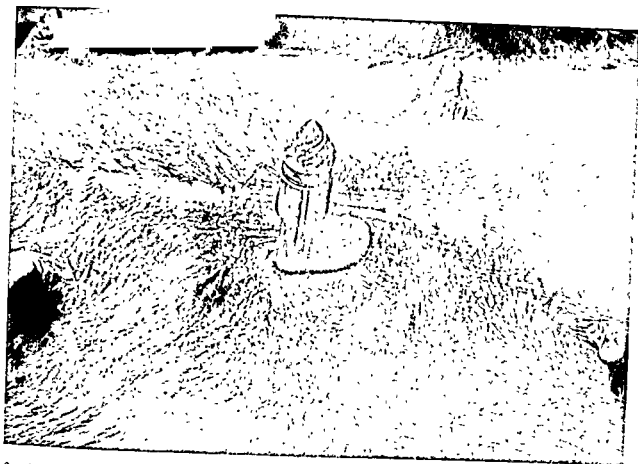


Fig. 2.—Photograph of the abdomen of a Dragstedt fistula dog. The "conical tip" has been partially screwed into the stainless steel cannula for illustrative purposes.



Fig. 3.—Photograph of the abdomen of an Inlow fistula dog. Note that the pancreatic duct orifice occupies the center of the picture. At the time the photograph was taken this dog had secreted pancreatic juice continuously for a period of more than six months.

that the "ground" surfaces face the operator and the *under* surfaces of the severed ends of the duodenum are adjacent. A continuous Connell suture is laid between these duodenal surfaces. A No. 1 silk suture, on a fine curved intestinal needle, is begun on the mesenteric border of the proximal bowel close to the forceps and ended at the antimesenteric border of the distal bowel segment. The Martzloff forceps are rotated to bring the "unground" surfaces of the forceps together and the cut ends of the bowel directly opposite. Another Connell suture is placed across the forceps, starting on the mesenteric border of the distal bowel close to the caudal forceps and finishing above the rostral forceps at the bowel's antimesenteric border. The antimesenteric sutures are grasped in one hand, the mesenteric ones in the other, and moderate tension is maintained while the forceps are removed by the assistant. The cut ends of the bowel are drawn together snugly and sutures are tied after inspection shows satisfactory bowel approximation. (Sutures should not be tied too tightly.) The anastomosis is manipulated to establish luminal patency and examined for leaks. A row of interrupted silk Halsted sutures is placed to reinforce the anastomosis. (The anastomosis must be tight because leakage is followed by peritonitis.)

The pouch and base of the cannula are draped carefully with omentum. Four interrupted linen sutures are passed through the pouch wall, 1 cm. in each direction from the cannula. A midline stab wound (opposite the junction of the middle and lower thirds of the abdominal wound) is made which is just large enough to admit the "conical tip" of the cannula that is brought through it. The linen sutures are placed through the abdominal wall about the cannula with a Reverdin needle; the sutures are tied tightly enough to hold the pouch against the peritoneum. This procedure,<sup>10</sup> which prevents intra-abdominal leakage and subsequent peritonitis by holding the pouch to the peritoneum, is a most effective one.

After washing the hands thoroughly, the abdomen is closed with clean instruments and catgut suture. The skin is sutured with interrupted linen and the wound sponged with tincture of iodine. No bandage is applied to the wound.

*Postoperative Treatment.*—For the first three to five days the animals are given no food or water. Intravenous Ringer's solution (100 c.c. per kg. body weight) is administered daily during this period. A padded high metal collar<sup>11</sup> prevents the animal from disturbing the cannula or the wound. As soon as pancreatic secretion appears, the dogs are placed in cages having wire bottoms in order to avert the development of pedal ulcers; such ulcers develop from prolonged contact with *active* pancreatic juice.

A soft diet is given about the fifth postoperative day; the pouch-anchoring sutures are removed at this time. Subsequently the diet consists of dog biscuits and milk. Sodium chloride (0.5 Gm. per kg. body weight per day) is added to the drinking water in order to replace the electrolyte lost by the dog through pancreatic secretion.

Collection of pancreatic juice, by means of a football bladder carried in an apronlike bag tied about the dog's abdomen, is initiated about the sixth postoperative day.

Postoperative complications are peritonitis and obstruction of the bowel. Peritonitis may result from a leak in the duodenal anastomosis or rupture of the duodenal pouch. Intestinal obstruction, which may occur in an animal possessing an intestine with a small lumen, is an unusual complication. The side-to-side anastomosis which would eliminate such obstruction, cannot be used for the high duodenal anastomosis.

#### THE INLOW FISTULA

Of the various pancreatic fistulas in which the major pancreatic duct is transplanted to the anterior abdominal wall, this preparation has proved most satisfactory in our hands. The two-stage operation is far superior to the single-stage one because of the lower postoperative mortality. The operation is superior to the method of Pavlov since the Inlow fistula can be prepared easily. The Inlow method has been modified in this laboratory to simplify the operative procedure and to secure a higher percentage of satisfactory fistulas.

*Operation. First stage.*—A 10 to 20 kg. dog is anesthetized with ether. A straight 10 cm. right rectus incision is made through the skin and subcutaneous tissue, starting just below the costal border, 2 cm. lateral to the midline. The subcutaneous tissue is separated from the fascia laterally to form a pocket which will receive the transplanted duodenum. The abdominal incision is completed along the original line, the duodenum is delivered into the wound, and the major pancreatic duct is located *vide supra*. The pancreas is freed from the duodenum 2.5 cm. above and below the duct after careful ligation of all blood vessels.

The transplantation of the duodenum beneath the abdominal skin is accomplished by closure of the fascia, muscle, and peritoneum, about the gut and pancreas adjacent to the pancreatic duct by four No. 2 chromic catgut-mattress sutures. The most rostral suture is passed between the bowel and pancreas about 3 cm. above the duct (which should lie about the center of the wound). The lowest suture is placed between the gut and the blood vessels at a region about 3 cm. below the duct. These sutures are placed so that the wound will fit snugly about the bowel where the latter enters the abdomen. The intermediate mattress sutures are placed *straight* through the pancreas near its free border at levels, just above and below the duct. This ensures the exteriorization of sufficient pancreas. (In placing the sutures the blunt end of the needle proceeds first to avoid blood vessel rupture.) The mattress sutures, with the exception of those passing directly through the pancreas, are tied tightly.

The duodenum is rotated to the right as far as possible and is fixed to the fascia beneath the lateral skin flap (catgut sutures between the rectus fascia and intestinal submucosa). This holds the gut and pancreas firmly and places the pancreatic duct in a *superficial* position at least 2.5 cm. lateral to the wound margin. After all hemorrhage has been controlled the subcutaneous tissues and skin are closed over the transplanted bowel with catgut and linen sutures.

*Second stage.*—After a four-week interval (to reduce hemorrhage at second operation) the transplantation of the major pancreatic duct is performed

under ether anesthesia and careful asepsis. The middle 6 cm. of the old skin wound is reincised, and the pancreatic duct and adjacent pancreas are located by cautious dissection. After careful mobilization and dissection of the duct to secure maximum length, the duct is severed partially at its intestinal junction. Fine silk, on a Carrel artery needle, is passed through the free lip of the duct. The duct is now completely severed from the bowel, and the defect in the latter is closed with a short continuous Lembert suture. Three additional sutures are placed through the end of the duct. A small stab wound, large enough to accommodate the duct, is made in the skin directly over the base of the duct (about 2.5 cm. from the edge of the incision). In order to reduce the tension upon the sutures between the duct and the skin, catgut sutures are placed between the duodenum and the cutaneous tissues on either side of the stab wound. *This anchors the skin firmly to the bowel.* The pancreatic duct is delivered easily through the stab wound and anchored *loosely* to the skin by the silk sutures. The original incision is now closed in the usual manner with catgut and linen.

*Postoperative treatment.*—The dog is placed in a cage having a wire bottom and his neck is fitted with a suitable padded collar to prevent his disturbing the wound. The dog can be fed as soon as his appetite returns. The diet consists of dog biscuits; sodium chloride is added to the drinking water. The duct sutures are removed about the fourth postoperative day.

Alkali dermatitis often develops in animals which secrete profusely. This is treated best by daily baths and applications of potassium permanganate 1:1,000. The chowchow, which has an alkali-resistant skin, requires less attention in this respect than other breeds (Fig. 3).

The pancreatic duct orifice is probed daily to avoid closure of the duct which occasionally occurs if this precaution is overlooked.

#### COMMENT

The Dragstedt fistula is an excellent preparation for those experiments in which pure pancreatic juice is not required. *This fistula has the advantages that the secretion is collected over the entire day; the animals remain dry and hence require little postoperative care; the normal duct-intestinal relationships are undisturbed.* The chief disadvantages of this fistula lie in the rather high incidence of postoperative peritonitis and the admixture of succus entericus, which contaminates the pancreatic juice in unknown quantities.

The Inlow type yields pure pancreatic juice which is collected, during an experiment, by a glass cannula introduced into the transplanted duct or by a glass funnel cemented by collodion to the abdomen adjacent to the duct. The fistula's chief disadvantages are alkali dermatitis as a result of profuse secretion and sensitive abdominal skin, closure of the duct through failure to probe it daily and inability to collect easily the entire daily secretion.

Observations of reasonable duration are made in both types of fistula on dogs which are trained to lie quietly upon a padded table. Drop-by-drop secretion is recorded by an automatic drop counter connected to the fistula. Extended observations are made upon animals placed in stocks.

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## CONTINUOUS VACUUM DISTILLATION\*

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THE isolation of bacterial specific substances from broth culture media, like many other procedures in organic chemistry, requires concentration of a large bulk to a small volume by means of vacuum distillation. It has become advantageous, therefore, to employ some time-saving modification in the usual apparatus.<sup>1, 2</sup>

A very inexpensive and efficient assembly is herein described and diagrammed.

## USE OF APPARATUS

After the system has been evacuated and the receiver *B* simultaneously cooled, the liquid from a stock bottle is transferred to the distillation flask by means of a siphon tube connected to the side arm *H*, *G*, *F*. Cocks *H* and *G* are opened, and when the desired amount has been transferred, they are closed. The siphon tube is then disconnected and the capillary tipped *I* is replaced. If frothing occurs, it may be controlled by manipulating *H* and *G* as described below. The distillation is now conducted until removal of the distillate is required. For removal, the three-way stopcock *D* is turned to evacuate catch flask *E*, and the screw clamp *J* is opened. By momentarily admitting air into the distillation flask via screw clamp *C* or cocks *H* and *G*, the distillate will be drawn through tube *A* into the catch flask. After drawing off the distillate, *J* is closed and *D* is turned to continue the distillation.

\*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.

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By this procedure distillation need not be interrupted. It is unnecessary to disconnect the vacuum pump and separate the receiver in order to pour off the distillate; reconnect and reseal broken points of attachment and start evacuating again. These are all time-consuming elements.

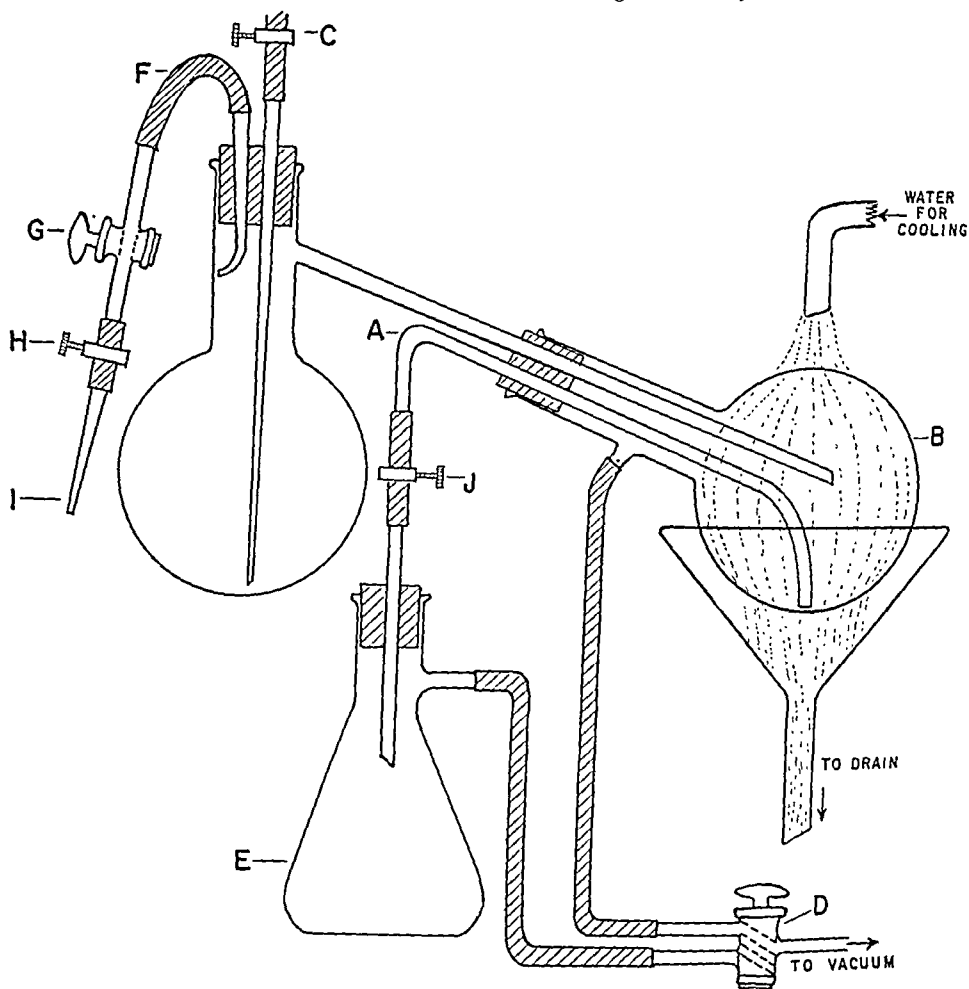


Fig. 1.—Distillation assembly.

#### DESCRIPTION OF APPARATUS

As shown in Fig. 1, a distilling flask is inserted into one of the holes of a two-hole rubber stopper, the usual capillary tipped glass tube. At the top of this is attached a rubber tube with a screw clamp *C*. The other hole is fitted with a bent glass tube *F* connected to a glass stopcock *G*. A piece of glass tubing drawn to a capillary or fire polished to a fine orifice *I* is connected to *G* by means of a rubber tube. Affixed to the latter is a screw clamp *H*. This modification may be used for two purposes: (1) As a froth controlling device<sup>3</sup> by using stopcock *G* as a coarse adjustment "valve" and screw clamp *H* as a fine adjustment. (The use of octyl or other higher alcohols as antifoam agents is eliminated.) By manipulating these "valves," the incoming air pressure prevents frothing over of the material into the receiver *B*. A little practice is



required to obtain optimum adjustment. While frothing is controlled, the distillation is evident by the appearance of a cloud formed in the receiver. The cold water or ice surrounding *B*, however, causes condensation of the cloud. (2) As an intake tube, the capillary *I* is removed and a siphon tube is attached. The other end of the siphon extends into the stock bottle containing the liquid to be distilled. For this purpose, it is advisable to fit the bent tube *F*, so that the end projecting into the distillation flask just touches the side of the neck. The incoming liquid then strikes the neck and flows smoothly down into the bowl of the flask, thereby preventing any additional froth. When the flask is filled to the desired level, the siphon tube is disconnected and the capillary *I* is replaced. Any frothing which occurs may be controlled.

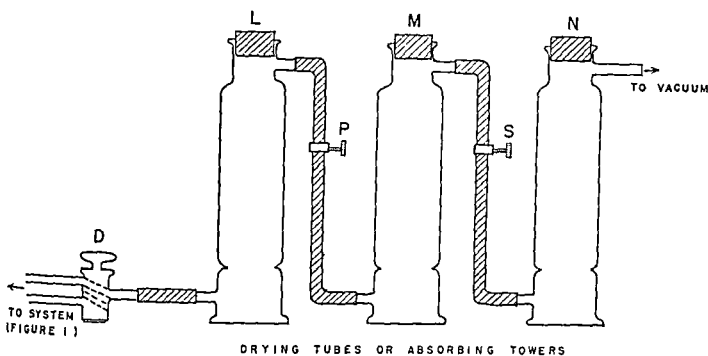


Fig. 2.

The condenser and receiver *B* also have a two-hole rubber stopper. One hole is for the side arm of the distillation flask which extends into *B*. Into the other hole is inserted a piece of glass tubing *A*. That end of the latter which projects into the receiver is bent, so that it just reaches the bottom of the distillate. At the other end is attached a length of rubber tube to which is affixed screw clamp *J*. From this, a piece of glass tubing extends into a one-hole rubber stopper in the mouth of catch flask *E*.

One of the parallel ends of a three-way stopcock *D* is connected to the catch flask, the other to the side arm of the receiver. The single end is connected to the drying tubes or absorbing towers and the vacuum pump.

A further improvement in the setup is obtained by arranging the drying tubes or absorbing towers, as shown in Fig. 2. When the first tube *L* becomes saturated with moisture and requires refilling with desiccant, it is merely necessary to close stopcock *D* and screw clamp *P*. Remove *L* and attach the rubber tube which connected the drying tubes *L* and *M* to stopcock *D*. *P* and *D* are then opened. *L* is recharged with desiccant and put back into operation again in its original position.

For maintaining sterile conditions, as in the distillation or concentration of biological products, e.g., sera, culture media, hormones, vitamins, etc., it is

necessary to connect a train of two or three bottles containing phenol or other bactericidal solution to *C* and *H*, the two points where air is admitted. The incoming air thereby is washed free from contaminants before entering the system. When the bactericide is already present in the liquid to be distilled, it is unnecessary to use the trains.

#### SUMMARY

A continuous vacuum distillation assembly is described and diagrammed.

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### THE MAZZINI TEST: A GREATER AID IN THE SERODIAGNOSIS OF SYPHILIS\*

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#### PRELIMINARY STUDIES

**I**N DEVELOPING his recently published flocculation test for syphilis, Mazzini<sup>1</sup> collected such information as could be obtained concerning those cases showing definite discrepancy in the results of the various tests employed in the Serological Laboratory of the Indiana State Board of Health. I have followed his work with great interest, and during the past several months have had the opportunity to add a few cases to the material he has collected and to employ his test in its first use in actual practice. It is with his permission that the material he collected has been analyzed and is herewith presented. The information was collected by means of questionnaires sent to the physicians in charge of the cases that showed definite discrepancies. As has already been stated,<sup>1</sup> the Mazzini test early demonstrated a greater degree of sensitivity in general than did the other tests we were using routinely. This was so apparent that in the cases of partial positives a slightly stronger result with this test was soon regarded as the usual finding and not as indication for a questionnaire. Questionnaires were not sent on specimens received marked for "treatment check."

In Table I are presented 502 cases in which there was discrepancy between the results of the various serologic tests. The cases classified as syphilitic are undoubtedly so according to the available information. Usually a definite statement indicating previous diagnosis of, or previous treatment for, syphilis was accepted as sufficient for syphilitic classification. In one or two cases sufficient information was given to question such a statement; these cases have been classified as doubtful with suggestive data.

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\*From the Bureau of Bacteriology and Pathology and the Lake County Survey Laboratory of the Indiana State Board of Health.

In general, the cases classed as doubtful with suggestive data are cases which might be regarded as diagnostic problems, with syphilis as a definite possibility. We have been "liberal minded" to the extent of considering that syphilis in a marital partner, congenital syphilis in a sibling, or a history of abortion or prostitution was suggestive of syphilis.

The cases listed as doubtful without suggestive data for the most part are so classed because of inadequate information. For example, a terse reply to the effect that the patient gives no history of syphilis is not sufficient evidence upon which to rule out the infection. The cases classed as nonsyphilitic are those rare cases in which sufficient information was obtained to exclude syphilis.

Table I is set up with the Mazzini test result as the basis of classification in the vertical column and the probable diagnosis in the horizontal spread. For example, 157 cases showed a 4-plus Mazzini reaction, of these, 97 were syphilitic, 41 were doubtful with suggestive data, and 19 were doubtful without suggestive data. Of the 157 cases, 18 gave negative Kline reactions; 81 gave negative reactions to the modified Kahn test; and 141 gave negative reactions with the complement fixation test. Not only did these negatives occur but also many of the other specimens gave only partial positives with each of the three tests; necessary condensation of the original table makes it impossible to show this here.

TABLE I

CASES SELECTED BY MAZZINI SHOWING DISCREPANCIES IN THE RESULTS OF VARIOUS SEROLOGIC TESTS FOR SYPHILIS. (Selected From Approximately 190,000 Specimens Examined.)

TEST RESULT	TOTAL NO.	PROBABLE DIAGNOSIS FROM AVAILABLE DATA:			
		SYPHILITIC	DOUBTFUL		NONSYPHILITIC
			WITH SUGGESTIVE DATA	WITHOUT SUGGESTIVE DATA	
Mazzini 4-plus	157	97	41	19	-
Kline negative*	18	14	4	-	-
Kahn negative†	81	54	18	9	-
C. F. negative‡	141	90	35	16	-
Mazzini 3-plus	217	145	46	26	2
Kline negative	62	49	9	4	-
Kahn negative	166	109	34	22	1
C. F. negative	207	138	43	24	2
Mazzini 2-plus	76	36	32	7	1
Kline negative	48	27	17	3	1
Kahn negative	66	31	29	5	1
C. F. negative	74	36	30	7	1
Mazzini 1-plus	11	3	4	3	1
Kline negative	2	1	1	-	-
Kahn negative	5	2	2	-	1
C. F. negative	10	3	3	3	1
Mazzini negative	41	9	9	22	1
Kline negative	4	2	1	1	-
Kahn negative	25	3	7	15	-
C. F. negative	34	5	8	20	1
Total cases	502	290	132	77	5

The tests, as performed in the Indiana State Board of Health Serological Laboratory, received the following rating in the 1938 evaluation survey conducted by the United States Public Health Service and the American Society of Clinical Pathologists:

\*Kline diagnostic test: sensitivity 82.4 per cent, specificity 100 per cent.

†One tube modification of the Kahn standard test sensitivity 82.4 per cent, specificity 100 per cent.

‡Complement fixation (Moon-Wassermann) technique. sensitivity 81.9 per cent, specificity 100 per cent.

## LAKE COUNTY (INDIANA) SYPHILIS SURVEY

In the Lake County Survey Laboratory the Mazzini and the Kline diagnostic tests are done on all specimens. Kahn standard tests are done only on those which show some degree of positive reaction with the above tests. The Kahn antigen used in this series was obtained from the University of Michigan Hospital.

The tests to be presented in this series represent 10,253 blood specimens. This total number classified by test and result are shown in Table II.

TABLE II

TEST RESULT	MAZZINI	KLINE	KAHN*
4-plus	1,491	1,010	540
3-plus	402	372	256
2-plus	219	344	264
1-plus	227	444	350
Negative	7,884	7,777	839
Not done	30	306	8,004
Total	10,253	10,253	10,253

\*Least false impression is left here, please note analysis of data section concerning this table.

Since the use of the Mazzini test in the Survey Laboratory represented its first use in actual practice and since the study presented in the preliminary studies seemed to indicate that the stronger reactions were satisfactorily specific, we were anxious to guard against the possibility of syphilis being falsely diagnosed as the result of an unsupported weak positive reaction with this test and to learn more of the significance of these weak positive results. Whenever such a reaction was encountered a questionnaire was sent to the physician in charge of the case, asking whether any evidence could be found in the history or physical examination to support or to contradict the result of the serologic examination. Questionnaires were not sent on specimens marked "for treatment check." One hundred eighteen of these questionnaires were sent out; 76 (64.3 per cent) were returned. The results are analyzed in Table III.

TABLE III

CASES SELECTED AT THE SURVEY LABORATORY; SPECIAL INTEREST IN WEAK POSITIVE RESULTS OF SEROLOGIC TESTS FOR SYPHILIS

OF SEROLOGIC TESTS FOR SYPHILIS						
TEST RESULT	TOTAL NO.	PROBABLE DIAGNOSIS FROM AVAILABLE DATA				NO. IN GROSS DIAGNOSTIC SERIES
		SYPHILITIC	DOUBTFUL		NON-SYPHILITIC	
			WITH SUGGESTIVE DATA	WITHOUT SUGGESTIVE DATA		
Mazzini 4-plus	3	1	1	-	1	1091
Kline negative	-	-	-	-	-	
Kahn negative	1	-	1	-	-	
Mazzini 3-plus	8	5	1	2	-	283
Kline negative	-	-	-	-	-	
Kahn negative	5	3	1	1	-	
Mazzini 2-plus	22	9	9	4	-	162
Kline negative	6	2	3	3	-	
Kahn negative	16	5	7	4	-	
Mazzini 1-plus	43	6	14	20	3	168
Kline negative	28	4	9	14	1	
Kahn negative	35	5	11	16	3	
Total	76	21	25	26	4	9228

## ANALYSIS OF DATA

Table I is largely self-explanatory; the point of chief interest is that the Mazzini technique detects more cases of syphilis than do the other tests under comparison. Particular attention is invited to the group of negative Mazzini reactions, where it is evident that this test was negative in cases which gave false positive reactions with the Kahn and Kline tests and even with the complement fixation test. Although not so clearly shown here as in the original unabridged table, the test also gave a certain number of false negative reactions which were detected by one or more of the other tests.

Table II illustrates the fact that while there is fairly close correlation between the number of negatives with the Mazzini and Kline tests (within 1 per cent of the series), the Mazzini test has taken many of the partial positives out of the doubtful range into the positive range of 3-plus and 4-plus. The apparent discrepancy between the number of positives and the number of Kahn tests done is explained partially by insufficient serum for the Kahn test and partially by the fact that for the first few weeks of operation of the Survey Laboratory, Kahn tests were not done there but positive sera were relayed to the State Board of Health Laboratory, Indianapolis, for Kahn and complement fixation tests, thus supplying a close interlaboratory check until we were assured that our results were satisfactory.

Table III is set up exactly as was Table I and represents the cases out of the series in Table II upon which questionnaires were sent out and returned; the additional column at the right indicates the total number of similar results in the diagnostic series of the gross run. Sixty-five 1-plus and 2-plus Mazzini cases represent a sampling of 19.6 per cent out of the 330 similar reactions in the gross diagnostic series. Twenty-two 2-plus Mazzini cases represent a sampling of 13.6 per cent out of 162 similar reactions in the gross diagnostic series. Forty-three 1-plus Mazzini cases represent a sampling of 25.6 per cent out of the 168 similar reactions in the gross diagnostic series. Calculation of the percentage of false positive reactions may be made according to the following formula:

$$\frac{\left( \frac{(N_s + D_s) \cdot S_g}{T_s} \right)}{T_g} = \text{Per cent of false positives,}$$

where:

- $N_s$  = nonsyphilitic cases in sample
- $D_s$  = doubtful cases in sample
- $T_s$  = total cases in sample
- $S_g$  = similar reactions in gross diagnostic series
- $T_g$  = total number in gross diagnostic series

The 2-plus Mazzini reaction was falsely positive to the extent of 1.03 per cent if all of the doubtful cases are admitted as false positives, or to the extent of 0.31 per cent if the cases classified as doubtful with suggestive data are not included. The median of this range is 0.67 per cent.

The 1-plus Mazzini reaction was falsely positive to the extent of 1.56 per cent to 0.93 per cent, or a median of 1.24 per cent.

For the combined 1-plus and 2-plus reactions, the so-called doubtful range, the per cent of false positives was from 1.24 to 2.59, with a median of 1.9. This

means that in this series the Mazzini test exhibited a specificity of approximately 98 per cent in the so-called doubtful range.

#### DISCUSSION

This study was undertaken primarily to increase our own knowledge of the dependability of the Mazzini test and has been pursued in an attempt to answer inquiries concerning its specificity. It is unfortunate that the data are not complete, so that the specificity for the so-called positive range of 3-plus and 4-plus might have been calculated. It logically would be much greater than in the range of 1-plus and 2-plus. The cases in Table I do not represent a true sampling, but include only those reactions which apparently were likely to reveal faults in the test. In view of this, it is believed that Table I indicates a satisfactory specificity. The calculation of specificity in the lower range is believed to be statistically sound and quite satisfactory for that range.

Specificity has been of great interest to pathologists and serologists. The clinician is more interested in what a given result, especially in the partial positive range, means in regard to his particular patient. Reference to Table III, which excludes treatment check examinations and deals only with the diagnostic series, shows that on the basis of a 2-plus Mazzini test, without knowledge of additional tests, the probability is approximately 1 in  $1\frac{1}{2}$  that the patient has syphilis, or 3 in  $3\frac{3}{4}$  that he presents a diagnostic problem with syphilis as a definite possibility. Similarly, the unsupported 1-plus Mazzini reaction indicates a probability of 1 in 7 that the patient has syphilis, or 1 in 2 that he presents a problem in the differential diagnosis of syphilis. Support by one or both of the additional tests definitely increases these probabilities. It was repeatedly found that only after serologic examination had shown some degree of positive reaction was a complete venereal history obtained.

It is believed that the data presented supply adequate support of our policy of reporting the various tests in terms of 4-plus, 3-plus, 2-plus, 1-plus, or negative, with the understanding that 1-plus and 2-plus are considered doubtful. The confusion of the clinician, sometimes claimed to result from such reports, should not (nor does it in our experience) for long exceed his competency.

In this analysis and discussion no attempt has been made to draw inferences concerning the indications for treatment or the continuation of it. Such a study would belong in the field of syphilology. In that respect reference is made to recent publications of Kolmer<sup>3, 4</sup> and Stokes.<sup>5</sup>

It is interesting to notice that in attempting to classify the cases of Table I as to diagnosis, difficulty was occasionally encountered, either because of a definite diagnosis of some condition other than syphilis or because of a differential diagnostic problem involving some condition other than syphilis. Such conditions included tuberculosis 8; carcinoma 7 (cervix uteri 2, liver 2, colon 1, lung 1, and carcinomatosis from teratoma of the testicle 1); pneumonia 4; hyperthyroidism 3; peptic ulcer 2; subacute bacterial endocarditis 2; Sydenham's chorea 2; mononucleosis; pneumococcal meningitis; typhus; empyema; Vincent's angina; acute rheumatic fever; staphylococcus otitis media, mastoiditis, and meningitis; and a nonpulsatile tumor in the midzone of the right chest. Incidentally, although not included in this series, "Rocky Mountain"

spotted fever has also given rise to the same difficulty in some cases. It is expected to deal further with the significance of these observations at a later time.

The serologic findings of the Lake County Survey Laboratory have been mentioned only from the standpoint of their bearing on evaluation of the Mazzini test; further analysis of their broader significance is reserved for a later study.<sup>6</sup>

During the past few years there has been an increased stimulus for careful scrutiny of results obtained in making serologic tests for syphilis. Increasing sensitivity has invoked vehement protests that false positives must necessarily follow. Two schools of thought have become even more sharply divided. One group believes that specificity must be safeguarded by maintaining an insensitivity of approximately 30 per cent. The other group, while recognizing the false position reaction as the paramount error of serologic technique, believes that the primary purpose of any such test is to furnish diagnostic aid in the detection of syphilis where it exists. To this end, they strive for maximum sensitivity consistent with 100 per cent specificity. The ideal is admittedly difficult to attain. In practice any test will give occasional false positives for biological reasons (that is, aside from technical faults). Therefore, in a large series of tests by any technique the specificity of 100 per cent may be approximated but not achieved. Specificity above 99 per cent for the so-called "positive" reports is, I believe, now generally accepted as satisfactory. Moreover, "all signs fail" when patients suffering from certain diseases are included in evaluation material.<sup>2</sup> Until more is known concerning the nature of "syphilitic" reagin, it is unlikely that we can do better than to recognize that such nonspecific results occur. This means, of course, that despite all talk of specificity the diagnosis of syphilis is, and must remain, a problem for medical judgment and must not depend entirely upon any serologic technique. This point cannot be too strongly emphasized. It would seem that a failure to appreciate this has caused undue alarm about the perils of high sensitivity.

#### SUMMARY

An analysis of 502 case studies (by the questionnaire method) collected in preliminary attempts to evaluate the Mazzini flocculation test for syphilis is presented. In addition, the results of the examination of 10,253 blood specimens, representing the first use of the test in actual practice, are presented. An analysis is made of a 19.6 per cent sample of the so-called doubtful reactions of the diagnostic series only. Calculated upon the basis of this sample, the test in this series maintained a specificity of approximately 98 per cent within the doubtful range. It is pointed out that within the so-called positive range of 3-plus and 4-plus, the specificity is expected to be much greater, and that the preliminary studies seem to indicate this, although calculation of this value is not attempted because of incomplete data.

The data presented indicate that the Mazzini test is more sensitive than the tests with which it is compared and is interpreted as indicative of a satisfactory specificity.

It is pointed out that a knowledge of specificity does not indicate the significance of a doubtful (or even of a positive) reaction in a given patient. An attempt is made to answer the latter question as it concerns the Mazzini test.

It is pointed out that false positives may occur with any serologic test for syphilis and that the diagnosis of syphilis should remain a problem for medical judgment.

#### CONCLUSIONS

The Mazzini flocculation test offers a greater aid in the serodiagnosis of syphilis than do certain other tests in common use.

Multiple tests are preferable to any single test.

Appreciation is expressed for the technical assistance of Miss Marie Martin and Mrs. Ann Walker; for the cooperation of the physicians of Indiana and especially of Lake County, without which this study could not have been made; and for the assistance of Mr. Kenneth W. Revell and his staff, of the Central Tabulating Unit of the U. S. Public Health Service, in tabulating a portion of the data.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**SULFANILAMIDE**, Studies on the Mechanism of the Action of, Lockwood, J. S., and Lynch, H. J. A. M. A. 114: 935, 1940.

Sulfanilamide has a bacteriostatic and limited bactericidal action in vitro on hemolytic streptococci, staphylococci, pneumococci, and colon bacilli. The magnitude of this effect is dependent principally on (a) the concentration of the drug, and (b) the concentration of "peptone" in the culture media.

"Peptone," as used in this paper, connotes any product of protein digestion whether prepared artificially in vitro or through the operation of natural enzymatic processes in vivo.

It is the author's belief that sulfanilamide acts by interfering with the nutritional requirements of susceptible bacteria and that the bacteria can die out in a phagocyte-free environment through starvation and autolysis.

The addition of peptone to media such as serum, which are deficient in nitrogen easily assimilable by bacteria, supplies such an excess of nitrogenous material that the bacteriostatic action of sulfanilamide is to a large degree inhibited.

**JAUNDICE**, Obstructive, The Renal Lesion in, Thompson, L. L., Frazier, W. D., and Ravdin, I. S. Am. J. M. Sc. 119: 305, 1940.

Except for the fact that their patients had a high incidence of albuminuria and a number of high blood urea nitrogen figures, the authors' clinical and experimental studies strongly confirm Elsom's observations on renal changes in hepatic disease.

A pre-existing renal lesion is intensified by a secondary "cholemic nephrosis." The high incidence of glomerular changes in the patients that died suggests that a pre-existing renal lesion may have been present. This is strongly suggested by the fact that these changes predominated in elderly patients.

If no previous renal injury exists, the evidences of "cholemic nephrosis" disappear rapidly following release of the obstruction. The major injury in this disorder is confined to the tubules.

The nephrosis associated with bile duct obstruction may be a determining factor in the final outcome and should not be looked upon as an inconsequential complication.

**VITAMIN C**, Effect of Renal Retention of, on Saturation Tests, Ludden, J. B., and Wright, I. Arch. Int. Med. 65: 151, 1940.

A modified five-hour vitamin C saturation test to compensate for the error due to variations in renal function is proposed.

Twenty-four-hour studies of the urinary excretion and plasma concentration of vitamin C after an intravenous test dose of 1 Gm. of ascorbic acid are reported for patients with a wide range of saturation levels and with various degrees of renal insufficiency.

Except for patients in the uremic state, the twenty-four-hour excretion appears to give a satisfactory index of the state of vitamin C nutrition. The excretion during shorter test periods gives false low values for patients with retarded vitamin C excretion. Patients with impaired renal excretion of vitamin C may give no other evidence of renal insufficiency.

A correlation exists between (1) the percentage of the five-hour output excreted during the first one and one-half hours after the test dose, and (2) the percentage of the

twenty-four-hour output excreted during the first five hours. In view of this, a formula is presented by which the actual twenty-four-hour output may be satisfactorily predicted through the use of data on urine specimens obtained one and one-half and five hours after the test dose.

The proposed test, in experience, gives a more reliable estimation of the actual state of vitamin C saturation than any other method available at this time.

In addition, an estimation of the renal function for vitamin C may be obtained through the analysis of data from this test.

The methods used follow:

Each subject was placed on a diet containing a minimum of vitamin C (less than 15 mg. daily) for at least twelve hours preceding the test and for twenty-four hours after the administration of the test dose. At 9 o'clock on the morning of the test the patient voided; six cubic centimeters of blood were drawn for plasma analysis and 1 Gm. of ascorbic acid, dissolved in 10 c.c. of physiologic solution of sodium chloride, were injected intravenously. Complete specimens of urine were obtained exactly one and one-half, three, and five hours after the injection, and were titrated immediately. The remaining specimen (nineteen-hour) was collected in tightly stoppered dark brown bottles, containing sufficient glacial acetic acid to bring the pH to 3, and kept at icebox temperature. This specimen was titrated immediately after the last micturition. For a number of patients with normal vitamin C excretion and for all patients with delayed excretion, the specimen obtained on the twenty-fourth hour was collected and titrated apart from the rest of the nineteen-hour specimen; blood was obtained from these patients at periods corresponding with the urine collections, and the plasma was analyzed for vitamin C.

The vitamin C in the urine was determined by a modification of Tillman's 2,6-dichlorophenolindophenol method and in the plasma by the sodium tungstate precipitation method of Farmer and Abt. (Duplicate plasma determinations, using the metaphosphoric acid technique of Farmer and Abt, gave values that agreed within 0.04 mg. per hundred cubic centimeters.) In every instance the plasma analysis was done within thirty minutes after venipuncture, and suitable blanks were used in all titrations. Potassium cyanide was not used as a preservative, since previous studies have shown that it reduces the dye and produces erroneous values.

The following modification is suggested for the saturation test:

Have the patient omit breakfast on the morning of the test. Immediately after the patient has voided and discarded the preliminary urine, inject intravenously 1 Gm. of ascorbic acid dissolved in 10 c.c. of physiologic solution of sodium chloride. Collect urine specimen 1 exactly one and one-half hours after the injection, and specimen 2 exactly five hours after the injection. Titration should be done preferably immediately after each collection, but, if necessary, the first specimen may be preserved as outlined previously and titrated at the end of the test.

By means of the values obtained for specimen 1 (a) and the sum of those for specimens 1 and 2 (b), the predicted twenty-four-hour excretion, C, may be calculated according to the formula  $C = \frac{ab}{12.6a - 0.27b}$ . The value thus calculated may be termed the saturation index.

**RESPIRATORY TRACT, An Epidemic Disease of, Reimann, H. A., and Havens, W. P.**  
Arch. Int. Med. 65: 138, 1940.

An epidemic disease of the respiratory tract occurred in Philadelphia and elsewhere in the winter of 1939. Reports of similar outbreaks in many parts of this country and in Europe suggest that it may have been pandemic. Of a group of 813 persons in the personnel of the Jefferson Medical College and Hospital, 50 per cent were ill. The majority of patients (88 per cent) were ill with nasopharyngolaryngitis. Six per cent had tracheobronchitis in addition, and 6 per cent had tracheobronchopneumonia. In most of the severely ill patients the lungs were presumably involved by the infectious agent suspected without the agency of the usual varieties of bacteria. There were no serious complications,

and all the patients recovered. The disease resembles epidemic influenza in many respects, but it is caused by a different agent. It represents a clinical entity probably caused by a filtrable virus.

**SULFAPYRIDINE, Parenteral Administration of, Haviland, J. W., and Blake, F. G. Am. J. M. Sc. 119: 385, 1940.**

The authors have presented a method of administering sulfapyridine parenterally which they deem quite satisfactory. One disadvantage of the method might appear to be the relatively large volume of fluid which must be used in order to put the desired quantity of sulfapyridine into solution. On the other hand, the authors have demonstrated by actual trial that such volumes may be given safely to almost any patient when necessary. The method will maintain adequate therapeutic blood levels. It will provide adequate fluids, salt, and glucose at a time when they are often needed either because of persisting vomiting or inadequate intake in delirious or comatose patients. It can be given without fear of slough or other reactions, such as may follow the use of the extremely alkaline sodium sulfapyridine. Furthermore, it may be given into any part of the body where needed, such as the veins, the subcutaneous tissue, the pleural cavities, or the subarachnoid space.

The method follows:

A weighed amount of the powdered drug was poured into the desired volume of the liquid to be tested or used. This liquid had been brought to the boiling point just before the addition of the powder. The mixture was shaken vigorously until all the powder had gone into solution, usually for one or two minutes. (Occasionally it seemed expedient to heat the mixture again to the boiling point in order to facilitate complete solubility.) The solution was then allowed to cool for six to eight hours, or until it had reached room temperature. Portions of these mixtures were then analyzed for their sulfapyridine content. Therapeutically the solutions were generally cooled fairly rapidly to the desired temperature.

The solutions were observed to assume a pale golden color upon standing. Whenever sulfapyridine precipitated out, it seemed to carry more and more of the substance with it, so that after a day or two a considerable clump of powder could be visualized on the bottom of the flasks. However, when the solution was complete, the sulfapyridine remained dissolved for several days. Aerobic and anaerobic cultures of such fluids were found to be sterile.

**TRICHINOSIS, A Study of 23 Cases, Murphy, F. D., James, H. D., and Rastetter, J. W. Am. J. M. Sc. 119: 328, 1940.**

The following points summarize the safeguards that the authors believe should be used for the control of trichinosis.

1. Individuals by their food habits expose themselves to the disease; the public should, therefore, be informed of the danger of trichinosis. They should be taught emphatically that insufficiently cooked food containing any pork is dangerous. The consumer must be brought to realize that he is, in the last analysis, responsible for getting trichinosis.

2. As Pote points out, the least likely source of the parasite is pork products prepared under Federal and adequate municipal supervision. The authors agree with his theory that it is the unprocessed pork products, especially summer sausage, prepared without supervision in small local slaughter houses or on farms, that frequently cause trichinosis. The jurisdiction of public health organizations should be made to include these sources of pork products.

3. The packer and swine grower, as pointed out by Hall, have it within their power and should be given the responsibility of setting up safeguards against the consumption of infested pork, particularly by excluding the hog which is fed uncooked pork scraps.

4. The medical profession should keep in mind the possibility of the disease when unusual cases of "flu" or "grippe" do not respond readily to treatment. If the physician

suspects the disease, the diagnosis usually will be made readily. All cases should be reported to the health department and attempts made to discover the source of the parasite.

Finally, it is emphasized that while the immediate responsibility for contracting trichinosis rests upon the consumer, the United States Public Health Service should assume the chief role in protecting the public from this disease.

**IODINE, Blood, in Acne Vulgaris, Traub, E. F., and Emmet, R. Arch. Dermat. & Syph. 41: 506, 1940.**

A series of 58 patients were studied from the standpoint of the iodine content of the blood.

Forty-five patients suffered from acne vulgaris. Thirteen patients had miscellaneous cutaneous diseases and were used as controls. Twenty-two additional, apparently normal persons, not included in the afore-mentioned group of 58, were also used as controls.

In the control groups of patients, whose blood iodine was apparently normal, the average value was approximately 6 micrograms per hundred cubic centimeters. The average iodine content of the blood of the patients with acne vulgaris was 5.975 micrograms, which was approximately within the normal range.

A slight difference was noted between the iodine content of the blood of males and that of females, the values for the males averaging 5.3 micrograms per hundred cubic centimeters and those for the females 6.65 micrograms.

Differences in the iodine content of the blood of patients in various age groups were recorded.

The failure to find a higher blood iodine content in patients with acne vulgaris than in "normal" persons possibly indicates that (1) the role, if any, played by iodine may be qualitative or (2) the iodine may, in patients with this disease, be stored in the cutaneous tissues.

**MEASLES, Disseminated Giant Cell Reaction, a Possible Prodrome of, Stryker, W. A. Am. J. Dis. Child. 59: 468, 1940.**

A pathologic change in a 27-month-old child who died of pneumonia was the presence of multinucleate giant cells in the pulmonary alveoli, bronchi, sinuses, and medullary cords of the tracheobronchial lymph nodes, lumina, and walls of the bronchial mucous glands, interstitial connective tissues about the lymph nodes and mucous glands, spleen, and lymphatic tissues of the ileum. These cells appeared morphologically like those described as specific for the prodromal stage of measles. A history of a possible contact with measles was obtained. Attempts to demonstrate inclusion bodies were inconclusive. This case is not presented as a proved example of the appearance of disseminated giant cells in the prodromal stage of measles; the existence of similar cells in proved cases, however, suggests the possibility here.

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## CLINICAL AND EXPERIMENTAL

### SULFAPYRIDINE: STUDIES ON ABSORPTION AND EXCRETION\*

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SINCE the first of January, 1939, we have been using sulfapyridine† in the Louisville City and the Children's Free Hospitals. When we began to use it, little was known of its mode of action and its fate within the human body, the best method of its administration, its toxic effects, etc. We decided, therefore, to investigate such of these things as we could. Consequently, we have done blood and urine studies on a number of patients large enough to give us some information concerning those points. This report deals with the results of those studies.

#### MATERIAL

Sulfapyridine has been given to date to 131 patients, of whom 103 had pneumonia. On most of them, at least a few blood studies of sulfapyridine concentration have been made. Of this total number, 46 were studied thoroughly from the point of view of the concentration of the drug in the blood, its absorption and its elimination. Of the 46 cases, 38 had pneumonia and 8 had other conditions (1 empyema, 1 gonococcal arthritis, 1 typhoid fever, and 5 *Streptococcus viridans* endocarditis).

#### METHODS

The free and total sulfapyridine in blood and urine were determined by the method of Marshall. In the earlier studies the total sulfapyridine determinations were obviously in error, and are not used in this material. Later, when

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†The sulfapyridine was furnished through the generosity of the Merck Laboratories, Rahway, N. J.

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we came across Marshall's demonstration<sup>1</sup> that accuracy could be ensured by the use of ammonium sulfamate, especially when toluenesulfonic acid was used to precipitate the blood proteins, this method was followed and has been used ever since with results which have never given us occasion to suspect any inaccuracy. Throughout, we have had no reason to question the free sulfapyridine figures. Colorimetry was done with the Evelyn photoelectric colorimeter.

#### PROCEDURE

In 14 of the 46 cases the initial dose was 2 Gm. followed after four hours by 1 Gm. every four hours day and night. In the remaining 32 cases the initial dose was 4 Gm.; in 18 of these the second dose of 1 Gm. was given four hours later, and was then repeated every four hours day and night; in 14 cases the second dose was not given until eight hours later, after which it was continued every four hours as previously.

In 38 of the 46 cases blood was drawn for analysis one, two, three, and four hours after the initial dose. Thereafter, blood samples were taken at variable periods, but frequently at six, eight, twelve, and from twelve to twenty hours later. In the other 8 cases the first four hourly samples were omitted, but samples were taken every morning at 8 o'clock, so that in nearly every instance we have determinations made from thirty-two to forty-four hours after the initial dose. Since without exception the drug was given at 12, 4, 8, and 12 o'clock, on the hour, the 8 A.M. samples always represented the blood level four hours after the latest preceding dose.

Whenever possible to collect, 24-hour specimens of urine were sent to the laboratory every morning throughout the course of treatment. Since for one reason or another it was frequently impossible to collect all the urine every day in every case there were only 16 cases in which complete urine studies were possible. Collection of urine was continued until the drug had practically disappeared from it.

#### RESULTS

(a) *Absorption.* As all other workers who have studied this phase of the subject have observed, absorption of sulfapyridine is very irregular and unpredictable. Fig. 1 shows the absorption curves in the first 37 consecutive cases of this study. It can be seen at a glance that there is no uniformity in the shape or the height of the absorption curves from identical doses (e.g., Cases 9, 10, 11, and Cases 20, 21, 22, 23, and 33), nor is there any consistency of relationship in the absorption from 2 and from 4 Gm. (e.g., Cases 5 and 13, Cases 11 and 12, and Cases 31 and 32). It should be noted that neither Case 20 nor Case 29 had previously had either sulfapyridine or sulfanilamide, for this point was carefully investigated.

Fig. 2 shows the "scattering" of the individual values at one, two, three, and four hours after the administration of single doses of 2 and of 4 Gm. The lines connect the median points, giving what may be called typical curves. It will be noted that the curve following the 2 Gm. dose is even slightly higher than that following the 4 Gm. dose. It may be pointed out further, that if the

two highest readings for each period of the 4 Gm. dose were omitted, the general appearance of the two halves of the figure would be almost identical. Fig. 3 shows the typical curves (constructed from the median value for each period) carried on for a period of from thirty-three to forty-four hours after the initial dose. Here, too, the blood concentration of free sulfapyridine is higher following an initial dose of 2 Gm. than it is from 4 Gm., the maintenance dose being the same in both instances.

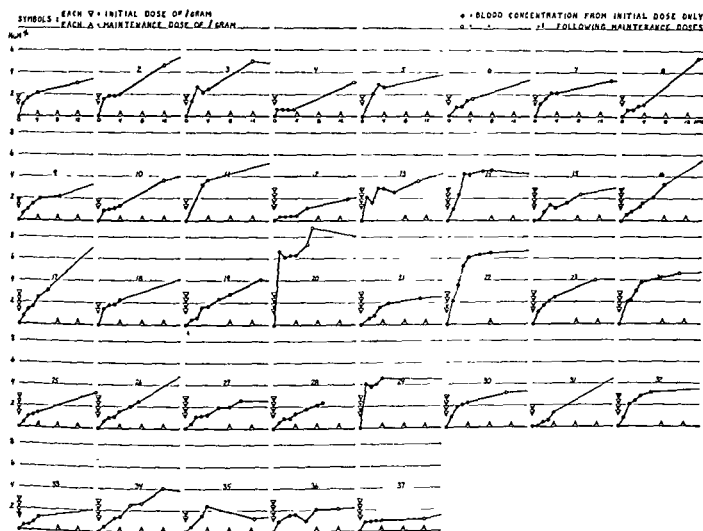
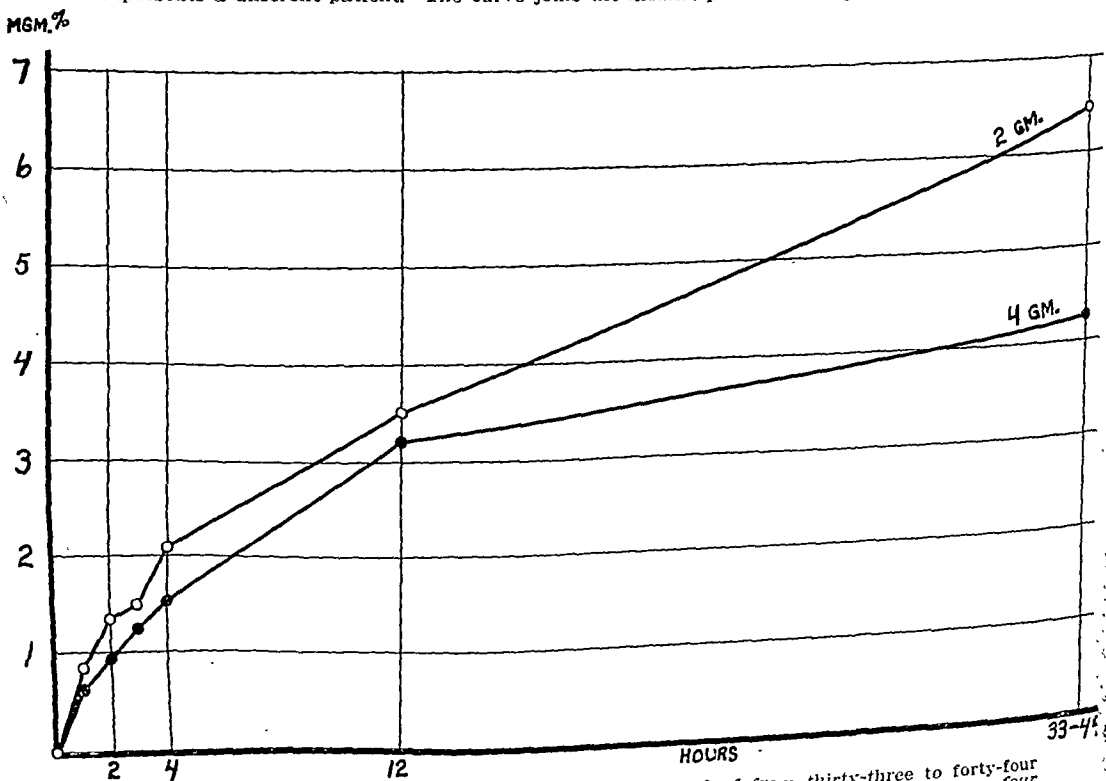
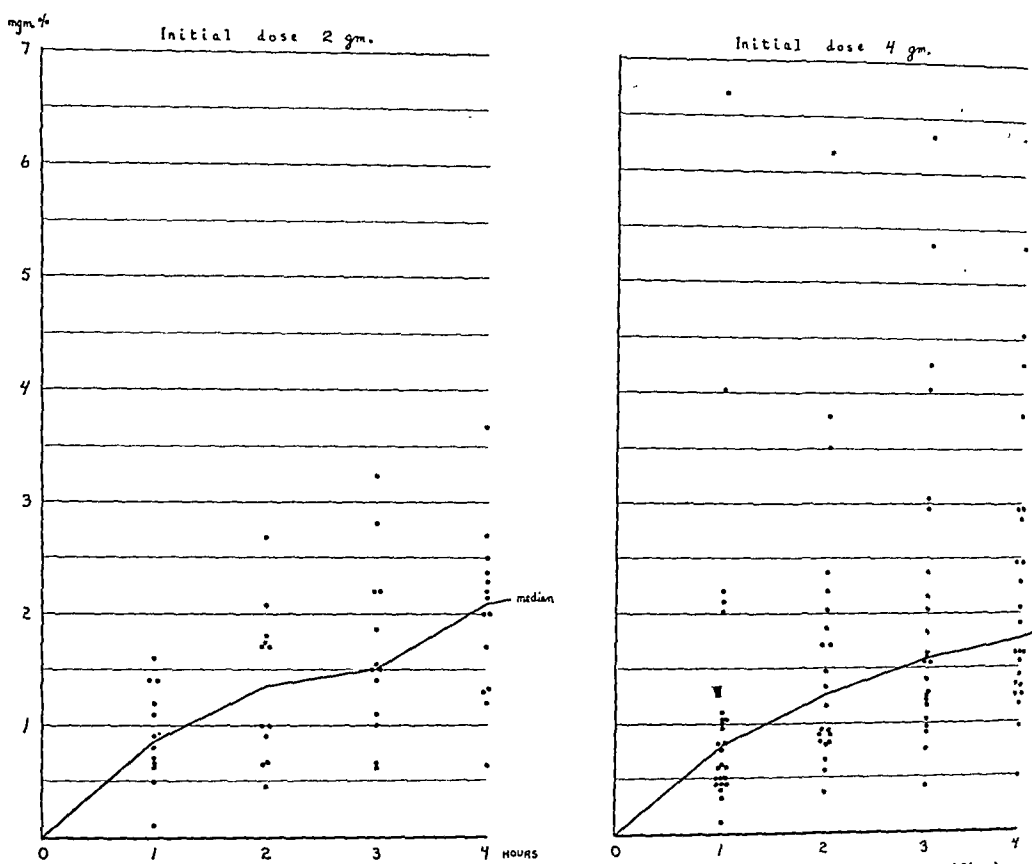


Fig. 1.—The absorption curves of free sulfapyridine in the first 37 consecutive cases. Each arrowhead represents an oral dose of 1 Gm., the downwardly directed ones indicating the initial dose and the upwardly directed ones, maintenance doses. The solid dots indicate the blood concentration figures from the initial dose only, and the open circles from maintenance doses as well. The thin line at the end of each curve points to the next concentration point, some time later than fifteen hours after the initial dose.

This paradoxical situation is very interesting and of practical importance. A logical explanation would lie in the assumption that the larger dose produced vomiting, thus preventing further absorption of the drug. As a matter of fact, vomiting followed the larger doses (67 per cent of the cases) more frequently than it did the smaller ones (45 per cent of the cases). In the entire series 58.7 per cent vomited. However, a study of Fig. 4 will show that even where there was no vomiting, the curve is still much higher following the 2 Gm. than that following the 4 Gm. dose. The same thing is also true where there was vomiting. We, therefore, cannot explain the unexpected discrepancy between size of dose and height of the concentration curve on the factor of vomiting; it is possible that a very large number of cases would have reversed this trend, but in the group from which these figures were obtained there were 6 vomiting and 8 non-vomiting cases who received 2 Gm. as an initial dose, and 21 vomiting and 11





nonvomiting cases who received an initial dose of 4 Gm. At any rate, it seems to us that the figures are impressive enough to lead us to recommend a routine initial dose of 2 Gm. rather than the larger one of 4 Gm. The probability of vomiting will be lessened, and the blood concentration is likely to be not only as high but possibly even higher from the smaller dose.

It is interesting to speculate as to the cause of vomiting following administration of sulfapyridine. There are only two probable explanations: a local irritating action of the drug on the stomach, and a central action on the vomiting center. Both theories have their proponents. However, Marshall's demonstration that the sodium salt of sulfapyridine given intravenously caused vomiting,<sup>2</sup> is strong evidence for the central origin of the symptom.

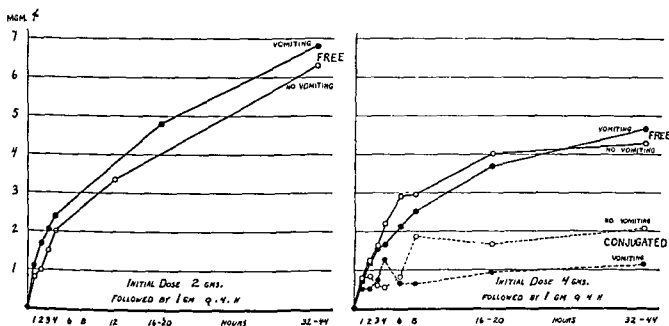


Fig. 4.—The relationship between vomiting and the concentration of free and of acetylated sulfapyridine in the blood. There were insufficient determinations of total sulfapyridine in the cases where the initial dose was 2 Gm. to compute the acetylated form.

The cause of the vomiting is not readily discerned from our investigations, but the fact that vomiting does not ensue following the initial large doses of 4 Gm. but only following subsequent smaller ones seems to us to argue against its purely gastric origin. Certainly a high concentration of the sulfapyridine in the blood is not by itself responsible, for with the higher concentrations following the smaller doses there is less probability of vomiting, as we have just noted. Moreover, the concentration during the period from twelve to forty-eight hours after the initial dose, when vomiting is most likely to occur, is about the same in the vomiting as in the nonvomiting cases when 4 Gm. have been given, and even higher for the vomiting than for the nonvomiting cases where 2 Gm. have been given. It has been suggested that the conjugated portion of the drug may be responsible for the vomiting. Our investigations do not bear this out, however, for as Fig. 4 shows, the level for the conjugated form is much lower where vomiting occurred than where it did not. One might consider the theory that perhaps there is a "critical vomiting level" for sulfapyridine in the blood were it not for the fact that the blood concentration at the time vomiting occurs varies tremendously in different individuals; however, had blood concentrations been determined at the time of onset of nausea, it is conceivable

that a fairly constant level might have been discovered. It has been our experience that frequently patients become nauseated or vomit early in the course of therapy, with relatively low blood levels; but if the drug is continued, the nausea lessens or disappears even while the blood concentration is rising. If vomiting is due to the action of the sulfapyridine in the blood on the vomiting center, it is probable that continued action by the drug eventually depresses the center after its initial stimulation.

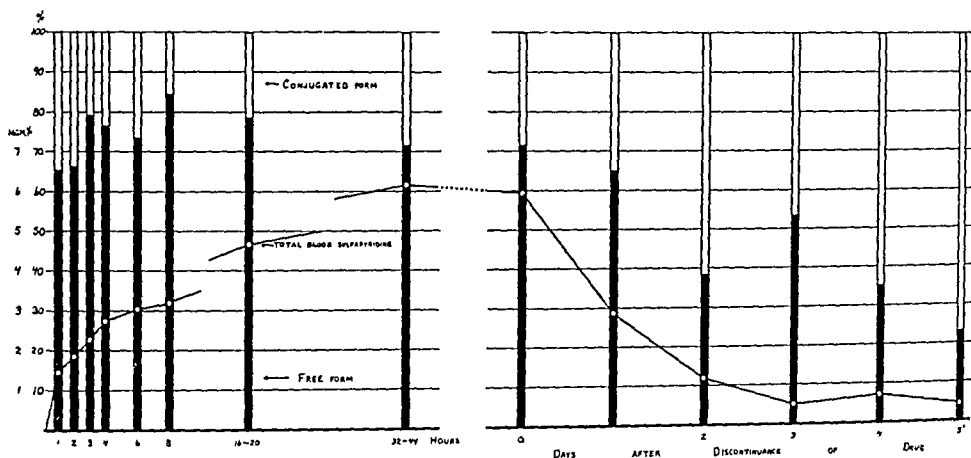


Fig. 5.—The blood concentration curve for total sulfapyridine throughout the stages of absorption, maintenance, and elimination, and the simultaneous relationship between the free and the acetylated forms. All the medians are from as many figures as were available.

(b) *Acetylation*. Acetylation or conjugation of the drug occurs very rapidly after it enters the blood stream—so rapidly that within an hour after it is given by mouth a very considerable proportion of it has already been conjugated (Fig. 5). In six or eight hours the maximum acetylation has occurred. During elimination the “free” form of the drug leaves the blood stream more rapidly than the “conjugated” form, so that during this phase the percentage of the acetylated form is much greater than at any other time. Table I shows this trend, the figures all representing median values and all following an initial dosage of 4 Gm.

TABLE I

THE ELIMINATION OF SULFAPYRIDINE: BLOOD CONCENTRATION STUDIES  
DURING THE ELIMINATION PHASE

DURING THE ELIMINATION PHASE							
	CONCENTRATION OF DRUG						
	ON DAY IT WAS STOPPED	DAYS LATER					
		1	2	3	4	5	
Free sulfapyridine	4.23	1.85	0.45	0.26	0.25	0.10	mg. %
Acetylated form	1.70	0.99	0.74	0.23	0.47	0.32	mg. %
Total sulfapyridine	5.93	2.84	1.19	0.49	0.72	0.42	mg. %

The degree of acetylation varies tremendously, however, not only from case to case but also from time to time in the same case. It may be as low as zero, or none at all, and as high as 80 per cent (exclusive of the “elimination stage,” when 100 per cent of the drug remaining in the blood stream may be

in the conjugated form). In general, though, from 10 to 45 per cent is in the acetylated form, the average being about 33 per cent. We could find no evidence for the statement sometimes made that the degree of acetylation depends to some degree on the concentration of the drug in the blood stream.

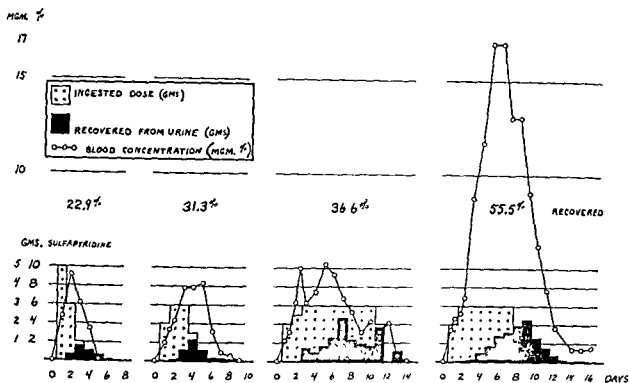


Fig. 6.—The relationship between the total amount of sulfapyridine ingested and the total amount recovered from the urine in 4 representative cases.

(c) *Elimination.* Following withdrawal of sulfapyridine by mouth, its elimination is fairly rapid (Fig. 5). Within three days most of it has been eliminated, but as long as five days after the last dose, easily measurable quantities can still be found in the blood stream. As noted above, the free form of the drug is excreted first and most rapidly, the conjugated form being excreted more slowly. Its recovery from the urine tends, as might be expected, to follow the blood level quite closely. After the drug is discontinued, the amount recovered from the urine parallels the diminishing blood level, so that it disappears from the urine at about the same time it does from the blood stream (Fig. 6). We have not noted the lag between the disappearance from the blood and from the urine spoken of by others. Generally speaking, when the blood level falls to about 0.4 mg. per 100 c.c., no more appreciable quantities can be recovered from the urine.

In 16 cases it was possible to collect the entire amount of urine passed during the whole course of sulfapyridine therapy. The amount of drug in the urine was measured quantitatively for the entire period. Table II records the findings. From this table it will be seen that the proportion of ingested drug which was recovered from the urine varied from 21.6 per cent to 55.5 per cent, the average being 29.8 per cent. Interestingly, when the cases are subdivided into vomiting and nonvomiting groups, the average excretion was still 29.8 per cent for both groups. It is presumed that the balance not recovered was not absorbed but passed unchanged through the gastrointestinal tract or was vomited. Hence, in general, one may say that about 30 per cent of the ingested drug is absorbed. In general, the free and the acetylated forms oc-

cur in the urine in about equal proportions; where there is vomiting, however, the free form is proportionately much lower (42.5 per cent) than where there is no vomiting (64.0 per cent), although the reverse is true for its distribution in the blood stream.

TABLE II

THE ELIMINATION OF SULFAPYRIDINE: QUANTITATIVE URINE STUDIES AND COMPARISON WITH AMOUNT OF DRUG INGESTED

CASES	AMOUNT RECOVERED FROM URINE			TOTAL ORAL DOSE	INGESTED DOSE RECOVERED
	FREE	PER CENT OF TOTAL	TOTAL		
Vomiting	Gm.		Gm.	Gm.	Per cent
12	10.40	46.8	22.20	40	55.5
16	13.11	64.5	20.31	73	27.8
18	15.25	54.3	28.07	94	29.8
19	2.02	56.6	3.57	12	29.8
20	2.20	60.1	3.66	13	28.2
29	2.26	38.1	5.94	18	33.0
34	5.63	25.2	22.33	61	36.6
36	1.68	28.2	5.95	19	31.3
40	1.71	18.8	9.08	42	21.6
41	1.10	26.6	4.13	18	32.0
No vomiting					
8	5.87	66.2	8.87	38	23.3
9	3.18	86.0	3.70	16	23.1
10	3.34	52.4	6.37	20	31.9
17	7.18	69.2	10.38	28	37.1
26	3.92	61.8	6.34	16	39.6
35	21.72	41.7	52.07	188	27.7
Median for vomiting group					29.8
Median for no vomiting group					29.8
Median for entire group					29.8

(d) *The optimum concentration of sulfapyridine.* Theoretically, it might be assumed that to obtain 100 per cent cures of pneumonia, the blood concentration should be above a certain figure, all other conditions being equal. Practically everyone who has written on this subject has reported a certain number of deaths in his series—not as many, it is true, as following other types of treatment, but still some deaths. One naturally asks if it is not probable that many, if not most, of those deaths were avoidable and were due to the fact that the concentration of sulfapyridine in the blood was too low to be effective. No figures concerning this point seem to be available. We have analyzed our own figures with this in mind.

In 103 cases of pneumonia of various kinds treated in the Louisville hospitals during the past season there were 6 deaths—a mortality rate of slightly under 6 per cent. These 6 cases include one patient who had advanced cardiovascular disease and who was already living "on borrowed time." It was not reasonable to expect any beneficial effects from sulfapyridine in this patient, but, nevertheless, she was given the benefit of the doubt and the case is included in this series. Two of the remaining 5 cases had extensive, bilateral bronchopneumonia, and the remaining 3 had lobar pneumonia.

The highest concentration of free sulfapyridine in the blood at any time in the fatal cases is compared with the highest concentration in the recovered cases in Table III, the figures being grouped over a 2 mg. per cent range. In

60 per cent of the fatal cases the concentration was less than 4 mg. per cent, and in 80 per cent, it was less than 6 mg. per cent, whereas in only 30 per cent of the recovered cases was the greatest concentration under 4 mg. per cent; 30 per cent had a maximum concentration between 4 and 6 mg. per cent, and 40 per cent had a maximum concentration over 6 mg. per cent. The obvious implication is that, although it is reasonable to expect a certain number of cures with a blood concentration of free sulfapyridine below 6 mg. per cent, yet to ensure the maximum number the concentration may well be over 6 mg. per cent.

TABLE III

DISTRIBUTION OF THE MAXIMUM CONCENTRATION FIGURES IN ALL CASES OF PNEUMONIA WHERE THERE WERE AT LEAST 3 DETERMINATIONS (FREE SULFAPYRIDINE)

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Recovered cases (57)	17 29.8%	17 29.8%	13 22.8%	10 17.6%
Fatal cases (5)*	3 60.0%	1 20.0%	1 20.0%	0 0%

\*One of the fatal cases had unreliable determinations

A fairer method of arriving at the optimum figure is to compare the maximum concentration in the fatal cases with that concentration in the recovered cases, *following which* the temperature remained normal and the patient went on to recovery, for in a few cases the blood concentration continued to rise even after the patient was getting well. In Table IV is recorded this comparison, the figures being based on as many cases as we had available for the preparation of the data needed. Here too, in only 30 per cent of the recovered cases was the concentration below 4 mg. per cent, about 30 per cent had a concentration between 4 and 6 mg. per cent, and 40 per cent had a concentration over 6 mg. per cent. These figures compare almost exactly with those in which the *maximum* concentration is taken because of the fact that it was in *only a few* cases that the concentration kept rising after the patient was on the road to recovery.

TABLE IV

COMPARISON OF THE MAXIMUM CONCENTRATION IN THE FATAL CASES OF PNEUMONIA, WITH THAT CONCENTRATION FOLLOWING WHICH THE TEMPERATURE REMAINED NORMAL AND THE PATIENT WENT ON TO RECOVERY

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Recovered cases (22)	7 31.8%	6 27.3%	7 31.8%	2 9.1%
Fatal cases (5)*	3 60.0%	1 20.0%	1 20.0%	0 0%

\*One of the fatal cases had unreliable determinations.

We may reiterate, then, that from our work it seems desirable, in order to reduce the mortality rate to the lowest possible figure, that the blood concentration of free sulfapyridine be maintained around at least 6 mg. per cent. It follows from this that in actual practice fairly frequent determinations of blood concentrations should be made, especially if the patient does not seem to be responding to treatment. If the concentration is not reaching a level of at least

6 mg. per cent and the patient is not improving, then the drug should be given parenterally, or the sodium salt should be used, whenever it becomes commercially available. While this precaution (frequent checks on the blood concentration) may seem a useless expense to the patient and may as a matter of fact actually not be necessary in the great majority of cases, yet for the sake of the exceptional cases it seems to us desirable to subject every patient to this routine. Our position in this respect seems justified since in the human being we are not yet as cognizant as we are in mice of the effectiveness of the drug in the various types of the pneumococcus.

TABLE V

COMPARISON OF CASES WITH AND WITHOUT BACTERIEMIA. THE DISTRIBUTION OF THE BLOOD CONCENTRATION FIGURES FOLLOWING WHICH THE TEMPERATURE REMAINED NORMAL AND THE PATIENTS WENT ON TO RECOVERY

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Positive blood culture (11)	4 36.4%	2 18.2%	5 45.5%	0 0%
Negative blood culture (11)	3 27.3%	4 36.4%	2 18.2%	2 18.2%

\*One patient had a *Staph. aureus* blood culture; the remainder had pneumococci.

TABLE VI

COMPARISON OF CASES WITH AND WITHOUT BACTERIEMIA. THE DISTRIBUTION OF FIGURES OF MAXIMUM CONCENTRATION AT ANY TIME

BLOOD CONCENTRATION MG. %	0-4	4-6	6-8	OVER 8
Positive blood culture (16)*	2 12.5%	4 25.1%	5 31.3%	5 31.3%
Negative blood culture (19)	7 36.9%	6 31.6%	4 21.1%	2 10.5%

\*Two patients had *Staph. aureus* blood cultures; the remainder had pneumococci.

*Bacteriemia.*—In an effort to determine the effects of different blood concentrations in cases with and without a blood stream infection, our figures were analyzed as outlined immediately above, the cases being divided into recovered cases with and without positive blood cultures. (Note: Of the fatal cases, 3 had positive blood cultures, 1 had a negative culture, and in 2 no blood was taken for culture.) From Table V can be seen the paradoxical fact that 55 per cent of the patients with bacteriemia had a normal temperature followed by recovery, even though the blood level was under 6 mg. per cent, whereas 64 per cent of the nonbacteriemic patients recovered under the same circumstances. On the other hand, when one considers the maximum concentration at any time (Table VI), it is seen that only 40 per cent of the bacteriemic patients recovered when the maximum concentration was less than 6 mg. per cent, whereas about 70 per cent of the nonbacteriemic patients recovered under the same conditions. From this it follows that it was perhaps not a bad thing that often the blood concentration kept on rising after the patient began to get well, for had it not he might not have recovered. These findings do not contradict our opinion as to a minimum blood level of 6 mg. per cent being desirable.

## SUMMARY AND CONCLUSIONS

1. In the Louisville hospitals sulfapyridine has been given to a total of 131 patients, of whom 103 had pneumonia. Of these, extensive studies were made concerning absorption, concentration in the blood, and excretion of the drug in 46 patients, of whom 38 had pneumonia.
2. The drug was administered orally in three ways: an initial dose of 2 Gm. followed by 1 Gm. every four hours; an initial dose of 4 Gm. followed four hours later by 1 Gm. every four hours; an initial dose of 4 Gm. followed eight hours later by 1 Gm. every four hours.
3. Absorption is very erratic, the height of the absorption curve being unpredictable from identical doses and from patient to patient, and there being no correlation between the size of the dose and the height of the blood concentration curve. In general, the blood concentration is higher following a dose of 2 Gm. than of 4 Gm.
4. Vomiting occurs in about 60 per cent of the cases, but is more frequent (67 per cent) following an initial dose of 4 Gm. than of 2 Gm. (45 per cent). For this reason and from the observation noted above, we recommend the routine employment of an initial dose of 2 Gm. rather than larger doses. Our work does not clarify the cause of the vomiting, but we feel that it is of central and not of gastric origin. Certainly it is not due to an increased concentration of the acetylated form, for in the vomiting cases the acetylated form of the drug was much lower than in the cases where there was no vomiting. It is likewise difficult to explain, on the basis of vomiting, why the concentration should be higher from the smaller dose.
5. Acetylation or conjugation of the drug occurs very rapidly in the body, about 35 per cent of it being acetylated within the first hour. Throughout the course of administration about 33 per cent acetylation is present, although the degree of acetylation varies from 0 to 100 per cent. During the elimination phase the degree of acetylation is much greater than at any other time. We found no evidence that the degree of acetylation bears any relation to the total concentration of drug in the blood.
6. After the withdrawal of the drug by mouth its elimination occurs rapidly at first, then more slowly, so that as long as 5 days later appreciable quantities can still be found in the blood and in the urine. The free form is eliminated more rapidly than the acetylated form. The drug disappears from the urine at about the same time it does from the blood. Quantitative determinations show that from 21 to 56 per cent of the drug taken by mouth can be recovered from the urine, the average being 30 per cent.
7. Of our 103 patients with pneumonia, 6 died. Of these 6 it was reasonable to hope for recovery in 5; of these 6, 60 per cent had a maximum blood concentration less than 4 mg. per cent and 80 per cent less than 6 mg. per cent. From this it is concluded that had it been possible to have raised the concentration in all the fatal cases to over 6 mg. per cent, some of them might have recovered. From this we make the generalization that in order to ensure the maximum recovery rate, frequent blood concentration studies should be made

in *every* case of pneumonia, and if the patient does not seem to be doing well and the blood concentration is below 6 mg. per cent, then the drug or its sodium salt should be administered parenterally.

8. Only 40 per cent of the cases with bacteriemia recovered with a blood concentration less than 6 mg. per cent, whereas in the cases with a negative blood culture, 70 per cent recovered under the same conditions.

We are indebted to Miss Beatrice Denny for her technical assistance.

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### STUDIES OF SULFANILAMIDE IN BLOOD AND URINE OF RABBITS INFECTED WITH BETA HEMOLYTIC STREPTOCOCCI\*

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SINCE the observation that sulfanilamide and related compounds are efficacious in the treatment of various infections, particularly those caused by the beta hemolytic streptococcus, many investigations have been carried out to determine the properties and actions of these drugs. At the time the present work was undertaken, however, none of the investigators had established a definite correlation between the dose of the drug and its level in the blood stream which should be maintained for clinical cure. The present investigation sought to supply this information, as well as to study the elimination of the sulfanilamide in the urine.

Infection was induced in rabbits by injecting beta hemolytic streptococci subcutaneously. Various methods of inoculation were tried and discarded because they failed to produce satisfactory foci with bacteriemia in the animals. The method finally chosen for the experiments utilized a mixture of 5 c.c. of an eighteen-hour blood broth culture and an equal volume of a 5 per cent preparation of mucin in salt solution. Two cubic centimeters of this suspension were injected subcutaneously through the shaved skin of the abdomen.

Considerable difficulty was experienced in producing a bacteriemia. Although the local lesions measured as much as 140 by 90 mm. and were elevated as much as 25 mm. and although they persisted two to three weeks, only 2 of 28 rabbits showed any streptococci in the blood stream. Since the disappearance of the bacteriemia could not be used as a criterion of successful

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treatment, we had to rely on the healing of the local lesion, return of temperature to normal, and recovery of the animal to adjudge the success of treatment.

Rectal temperatures were recorded twice daily and blood cultures were made daily until the animal died or recovered. Two cubic centimeters of blood were taken from the heart twice daily, the one at approximately the time when the blood level of the drug would be at the maximum, and the other at the time when this level would be at the minimum. On each of these specimens the amount of free and total sulfanilamide was determined by a method devised by Marshall.<sup>1</sup> In each of 7 sets of experiments 4 rabbits (weighing about 2 kg.) were studied; 2 animals were treated and 2 served as controls. The former were placed in metabolism cages and the urine was collected for twenty-four-hour periods. Determinations of free and total sulfanilamide were made on these urine specimens as long as the animals were under treatment. The method of analysis was that of Marshall, Emerson, and Cutting,<sup>2</sup> as modified by one of us (Hansen<sup>3</sup>).

For treatment, sulfanilamide was prepared in 0.25, 0.5, and 1 per cent solutions. Each of the treated rabbits in a series received 100 c.c. of one of these solutions twice in twenty-four hours through a stomach tube. The first daily dose was given in the morning, followed by the second dose about four hours later. The next treatment was not given until the next morning, twenty hours later.

Available data from studies on man, dogs, rabbits, rats, and mice<sup>2, 4-8</sup> indicate considerable differences regarding the time of administration of the drug and the attainment of a maximum blood level between human beings and lower animals, as well as between individual members of the same species. Furthermore, the free sulfanilamide is presumed to be more active therapeutically than the acetylated derivative. Obviously, it was necessary to obtain information relative to the maximum level of the sulfanilamide in the blood and also data concerning its conjugation before studying infected animals. Four series of uninfected rabbits were treated orally over periods of three days. Blood analyses were made as indicated below (Part I). This information was then used in choosing the time for taking blood specimens for study from the infected animals (Part II).

#### PART I. LEVEL OF FREE AND CONJUGATED SULFANILAMIDE IN BLOOD OF UNINFECTED RABBITS

Two rabbits were treated orally with 100 c.c. of a 1 per cent aqueous solution of sulfanilamide at 8 A.M. on two consecutive days. Blood specimens were obtained at regular intervals and on the morning of the third day. Curves I and II in Fig. 1 give the blood levels of the total and free sulfanilamide in these rabbits on the two consecutive days, and Curve III indicates the per cent of the conjugated drug. The points of interest are: (1) On both days and for both rabbits the maximum level of the drug (free and total) was obtained with the first blood specimen, two hours after the dose, and these values were approximately the same for both rabbits and were nearly equal on both days.

(2) The levels of the total and free sulfanilamide ran nearly parallel, dropping slowly during the first eight hours after the dose and falling to approximately one-tenth of the maximum value twenty-four hours after the dose. (3) The conjugated drug quickly reached a level which remained nearly constant during the first eight hours. (4) The trend of conjugation was decidedly different for the two rabbits; in rabbit 14 the rate of conjugation was slow, and in rabbit 15 it was comparatively rapid. This difference in the conjugation was clearly indicated two hours after the dose, but more noticeably after greater time intervals, so that at the end of eight hours the first rabbit had about 70 per cent of the drug in the free state, while the other had about 70 per cent conjugated. (5) Twenty-four hours after the dose the percentage of the relatively small amount of free and conjugated drug still in the blood was about the same for both rabbits. On the basis of these observations it would seem best to obtain blood specimens for maximum values two hours after the dose, or possibly sooner than this, but about two to four hours after the dose to show the trend of conjugation.

TABLE I

SULFANILAMIDE CONCENTRATION IN BLOOD OF NORMAL RABBITS ON THE THIRD DAY OF TREATMENT

(100 ml. of 1 per cent solution of sulfanilamide administered twice daily.)

TIME	HOURS AFTER DOSE	RABBIT 14					RABBIT 29				
		MG. PER 100 ML. OF BLOOD		PER CENT			MG. PER 100 ML. OF BLOOD		PER CENT		
		TOTAL S	FREE S	CONJ. S	FREE S	CONJ. S	TOTAL S	FREE S	CONJ. S	FREE S	CONJ. S
7:00 A.M.	19	43.5	32.2	11.3	74.0	26.0	7.0	1.35	5.65	19.3	80.7
8:00	1	60.6	51.3	9.3	84.3	15.7	29.8	23.8	6.0	79.9	20.1
9:00	2	52.6	43.5	9.1	82.7	17.3	29.8	22.0	7.8	73.8	26.2
10:00	3	54.8	42.5	12.3	77.6	22.4	27.0	19.4	7.6	71.8	28.2
12:00 M.	5	50.0	39.2	10.8	78.5	21.6	23.25	15.93	7.32	68.6	31.4
1:00	1	57.1	50.0	7.1	87.5	12.5	42.5	36.4	6.1	85.6	14.4
2:00	2	57.1	46.5	10.6	81.2	18.6	40.8	32.2	8.6	79.0	21.0
3:00	3	57.1	50.0	7.1	87.5	12.5	35.1	29.0	6.1	82.6	17.4
5:00	5	54.0	44.5	9.5	82.4	17.6	28.6	22.2	6.4	77.7	22.4
10:00 A.M.	22	-	-	-	-	-	2.42	Trace	2.42	0.0	100.0

However, in order to secure data, first, regarding the blood level and conjugation of sulfanilamide obtained with different dosages, and secondly, concerning the increase of the maximum blood level when the drug is administered more than once a day, 100 c.c. of a 1 per cent aqueous solution of sulfanilamide was administered by stomach tube twice a day on three consecutive days to several rabbits. Two animals were treated in the same manner with 0.25 per cent solution. Blood specimens were taken at regular intervals on the third day of treatment.

Table I presents data on two of three rabbits which received the 1 per cent solution. Several points are to be noted. (1) The blood level in rabbit 14 was very high on the morning of the third day of treatment, nineteen hours after the preceding dose, especially when compared with the values obtained with the same rabbit some three weeks earlier, when the level after single

doses dropped to from 2 to 3 mg. for total sulfanilamide (Fig. 1) and likewise when it is compared with rabbit 29 that received identical treatment. (2) After each administration of the drug the highest values for the total and free sulfanilamide were found in the first specimen of blood, one hour after the dose. (3) The increase in level for total drug was about the same for both rabbits after the first dose, and after the second dose it was again similar for one rabbit but much less for the other. (4) A new high value was observed for the blood level of the drug in one of the rabbits after the second administration of the drug. (5) The drop in the blood level was not very rapid in

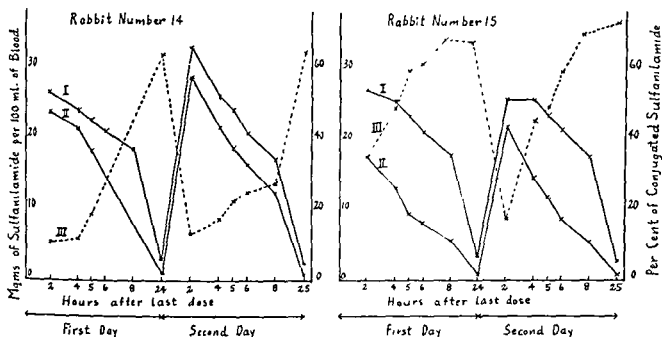


Fig. 1.—Sulfanilamide concentration in the blood of normal rabbits (100 ml. of a 1 per cent solution of sulfanilamide administered daily on two consecutive days) Curve I = Total sulfanilamide. Curve II = Free sulfanilamide. Curve III = Per cent of conjugated sulfanilamide.

the first five hours after administration of the drug. (6) The trend to conjugation was comparatively slight for both rabbits, but it was well established at the end of the third hour after a dose. It appears to be a consistent quality of a given animal: rabbit 14, for example, had manifested a similar capacity for conjugation when it was used before. Finally, the excretion appears to be unimpaired in one of the rabbits, and conjugation is complete for the drug which still remains in the blood twenty-two hours after the last dose.

Almost all the points enumerated hold also for the two rabbits which received the 0.25 per cent solution. The level for total drug appears to be roughly proportional to the dose. The highest levels were found in the first blood specimen, one hour after the dose. The trend to conjugation again was clearly indicated three hours after a dose, but conjugation was much higher than in the former set of rabbits, which indicated a characteristic difference between individual rabbits. The level for the total drug in the blood dropped slowly during the first five hours after a dose, but was down to approximately 1 mg. per cent for the total drug and only a trace of free drug twenty-two hours after the last dose.

In view of the fact that sulfanilamide is generally administered to man in the solid form and that it has a relatively low solubility, the drug was given

in gelatin capsules for a comparison with administration in solution. The drug was administered in divided doses at the rate of 1 Gm. per kilogram of body weight, twice daily, and on three consecutive days to 5 rabbits. Blood specimens were obtained at regular intervals for analysis on the first and third days and in addition on the morning of the second and fourth days of the experiment. Data from two of these rabbits are presented in Fig. 2.

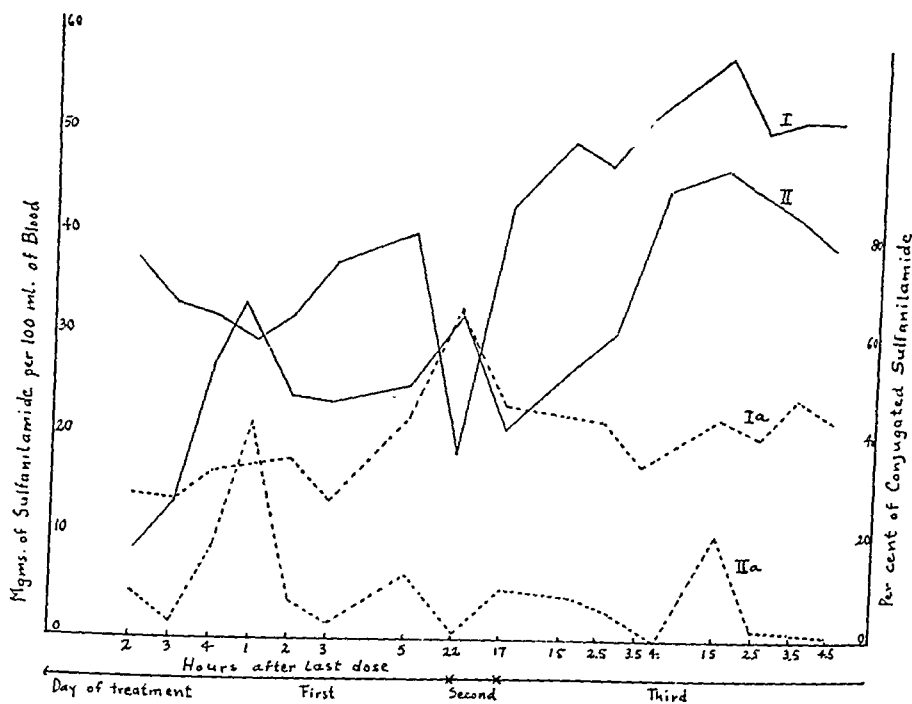


Fig. 2.—Sulfanilamide in the blood of normal rabbits. Administration in gelatin capsules twice daily on three consecutive days (1 Gm. of sulfanilamide per kilogram of body weight per day). Curves I and II = Total sulfanilamide for rabbits I and II. Curves Ia and IIa = Per cent of conjugated sulfanilamide for rabbits I and II, respectively.

The most characteristic feature to be noted in this series, as contrasted with administration of the drug in solution, was a marked irregularity in obtaining a maximum blood level. The actual level reached was very high, especially on the third day. In fact, there appeared to be a definite retention of the drug in both rabbits, judged on the basis of a high general level, and unusually high values for the morning specimens on the second, third, and fourth days, and on the recovery of the drug in the urine of one of the rabbits, which was 25.3, 35.8, and 30.6 per cent of the intake on successive days of treatment. Each of the rabbits appeared to have its own characteristic conjugation, the one having a high and the other a distinctly low capacity.

One of the rabbits was very ill and died at the end of the third day of treatment. Death was probably due to the effect of the drug. The other rabbit survived, although the blood level of the drug in this rabbit was markedly higher than in the rabbit that died. It seems that administration of the drug in solution results in a more uniform handling of the drug; it is also to be noted that in rabbits there may be an accumulation of the drug, which

may be a result of a temporary impairment of kidney function, a phenomenon we have repeatedly observed in infected rabbits treated with sulfanilamide.

#### SUMMARY AND CONCLUSIONS

1. Levels and distribution of free and conjugated sulfanilamide were studied in the blood of four groups of uninfected rabbits in order to determine maximum blood levels and trends to conjugation of the drug.

2. Maximum blood levels were obtained within one hour after administration of the drug, when it was given in an aqueous solution by stomach tube. But when the solid form was administered in gelatine capsules, maximum blood levels were obtained at irregular intervals after a dose.

3. The ability to conjugate the drug varies greatly with different rabbits. It appears to be characteristic for a given animal and is definitely established two to three hours after administration of the drug.

4. Blood levels of the drug increase with each administration and are roughly proportional to the size of the dose when the drug is administered in aqueous solution.

5. The amount of the drug in the blood drops slowly during the first five hours after a given dose.

#### PART II. SULFANILAMIDE IN BLOOD AND URINE OF RABBITS INFECTED WITH HEMOLYTIC STREPTOCOCCI

Seven series of rabbits were studied, each group consisting of four animals. They were infected and treated as previously described. Blood specimens were obtained twice daily by cardiac puncture: morning specimens, eighteen to twenty hours after the previous dose, to give minimum blood levels of the drug; and evening specimens, two and one-half and, in some instances, four and one-half hours after the last dose to give maximum blood level values, and also to indicate the tendency to conjugation. Elimination of the drug was determined by quantitative urinalysis. The drug was administered at the rate of 0.5 Gm. per day (approximately 0.25 Gm. per kilogram of body weight), 1 Gm. per day (about 0.5 Gm. per kilogram of body weight) in two series and 2 Gm. per day (about 1 Gm. per kilogram of body weight) in four series (Table II).

*Treatment With 0.5 Gm. of Sulfanilamide Per Day.*—In series<sup>1</sup> one of the controls and one of the treated animals recovered (Table II). The blood and urine data are given in Table III for the two treated rabbits over a period of eight days. For rabbit 37 the blood values for total and free sulfanilamide are given over a period of eighteen days (Fig. 3). This represents the first two periods of treatment. Rabbit 39 survived eight days, and rabbit 37 recovered after daily treatment over a period of four weeks.

Comparison of data shows similarities and differences which suggest certain conclusions with regard to effectiveness and toxicity of the drug. Elimination of the drug in the urine was nearly identical throughout the period of observation. The percentages of the conjugated form of the drug in the urine were almost identical. Likewise, the percentage of conjugated drug was uniformly

TABLE II  
RESULTS OF HEMOLYTIC STREPTOCOCCAL INFECTIONS IN RABBITS

DOSE PER DAY	SERIES	TREATED				UNTREATED		
		RABBIT NO.	DAYS TREATED	DAYS SUR- VIVED	CAUSE OF DEATH AND BACTERIOLOGIC FINDINGS	RABBIT NO.	DAYS SUR- VIVED	BACTERIOLOGIC FINDINGS AT AUTOPSY
0.5 Gm.	I	37	20	*	*	38	1	Local lesion— Hemolytic strep- tococci Blood—Hemolytic streptococci
		39	7	8	Drug Local lesion— Hemolytic strep- tococci	40	*	*
1.0 Gm.	II	34	2	7	Drug Local lesion— Hemolytic strep- tococci	33	*	*
		36	2	*	*	35	10	Local lesion— Hemolytic strep- tococci Liver abscess— Hemolytic strep- tococci
	III	41	17	*	*	43	*	*
		42	14	*	*	44	*	*
2.0 Gm.	IV	4	4	4	Accidental death during treat- ment	14	*	*
		10	5	7	Drug Local lesion— Hemolytic strep- tococci	15	*	*
	V	20	1	3	Peritonitis Local lesion— Hemolytic strep- tococci Peritoneal exudate Hemolytic strep- tococci	19	21	Local lesion— Hemolytic strep- tococci
		25	24	*	*	21	21	Local lesion— Hemolytic strep- tococci
	VI	24	18	37	Drug Cultures—No growth	27	30	Local lesion— Hemolytic strep- tococci
		26	7	10	Drug Local lesion— Hemolytic strep- tococci	28	*	*
	VII	29	11	*	*	31	4	Local lesion— Hemolytic strep- tococci
		30	5	*	Local lesion— Hemolytic strep- tococci	32	2	Multiple coccidial abscesses in liver Local lesion— Hemolytic strep- tococci

TABLE III

SULFANILAMIDE IN BLOOD AND URINE OF RABBITS: STREPTOCOCCUS INFECTION  
(Intake of drug 0.5 Gm. per day = approximately 0.25 Gm. per kg. of body weight.)

DAYS TREATED	TIME OF BLOOD SPECIMEN	RABBIT 37 (RECOVERED)					RABBIT 39 (DIED)				
		SULFANILAMIDE					SULFANILAMIDE				
		IN BLOOD		IN URINE			IN BLOOD		IN URINE		
		TOTAL MG. PER 100 ML.	PER CENT CONJUGATED	COLLECTION PERIOD HOURS	RECOVERY OF INTAKE PER CENT	PER CENT CONJUGATED	TOTAL MG. PER 100 ML.	PER CENT CONJUGATED	COLLECTION PERIOD HOURS	RECOVERY OF INTAKE PER CENT	PER CENT CONJUGATED
1	A.M.*										
	P.M.	7.98	40.2	24	57.8	84.1	10.13	26.8	24	-	-
2	A.M.	4.04	21.0				0.92	7.6			
	P.M.	7.73	60.2	24	72.5	89.3	5.27	39.3	24	60.9	89.4
3	A.M.	1.85	100.0				4.21	78.0			
	P.M.	7.94	63.0	24	77.5	81.8	8.97	67.0	24	75.6	88.0
4	A.M.	2.72	100.0				5.06	84.0			
	P.M.	9.75	67.6				11.00	70.7			
5	A.M.	No treatment					No treatment				
	P.M.			48	108.4	87.0			48	98.9	88.1
6	A.M.	3.20	20.6				4.39	100.0			
	P.M.	12.5	69.3	24	44.5	86.2	8.20	41.0	24	44.2	86.3
7	A.M.	5.43	51.0				6.10	54.8			
	P.M.	9.26	64.0	24	65.9	88.5	14.00	66.4	24	65.4	84.5
8	A.M.	2.91	66.0				12.28	81.7			
	P.M.	9.50	53.5	24	65.1	87.0	17.57	65.8	?	24.2	83.6
9	A.M.	4.2	60.0				Rabbit died during the night				

\*A.M. = morning specimen, eighteen to twenty hours after last dose (minimum blood level).

P.M. = evening specimen, two and one-half hours after last dose (maximum blood level).

higher in the urine than in the blood, which indicates that the conjugated form is more readily eliminated than the free drug. This is also true in human beings.<sup>3, 9</sup> They were similar in requiring two to three days to reach an approximately even level for percentage elimination of drug intake, which is explained by retention in the tissues during the initial treatment.<sup>2, 3</sup> That this is true is substantiated by further urine data from both animals. In a forty-eight-hour period elimination with intake limited to the first twenty-four hours, elimination is higher than the previously established level and even in excess of the intake. This indicates a drainage of the drug out of the tissues. On the succeeding days of treatment this is followed first by a lowered elimination rate, indicating retention by the tissues, and then by an increased elimination approximating the former rate. These similarities indicate that the kidneys functioned efficiently in both animals.

The picture of the blood was not so uniformly similar. The trend to conjugation tended to be higher for rabbit 39 than for rabbit 37. The amount of drug in the conjugated form was also generally higher, especially during the last several days, when it rose to above 10 mg. per cent in rabbit 39, while in rabbit 37 it seldom rose above 5 mg. per cent nor did the blood picture in rabbit 37 change appreciably during the later period of observation (Fig. 3). The difference in the amount of free drug in the blood was not marked at any time. On the whole then, while the amount of total drug was higher in rabbit 39 than in rabbit 37, especially during the last several days of its life, this difference was due essentially to a higher level of the conjugated drug.

It appears that the drug level in rabbit 37 was adequate for the control of infection, while in rabbit 39, in spite of a higher total drug level, due principally to a greater amount of the conjugated form of the drug, the infection could not be controlled. At autopsy the blood was sterile, but hemolytic streptococci were cultured from local lesions. It may be that toxins entered the circulation from the focus of infection and that death was due to toxemia. However, it seems to us that the toxic effect of the greater amount of the drug in the circulation, and especially of the conjugated form, was definitely a contributory factor in causing death.

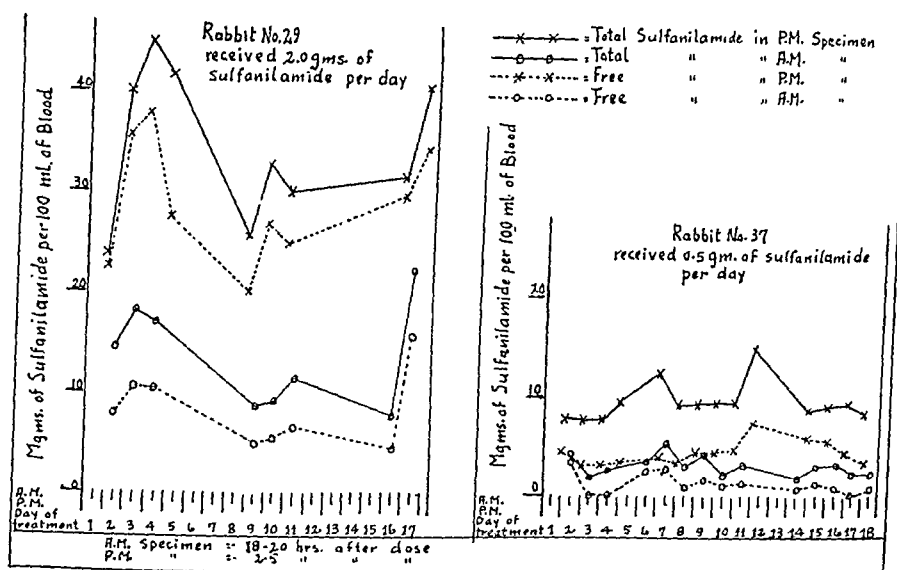


Fig. 3.—Maximum and minimum blood levels of total and free sulfanilamide in rabbits infected with beta hemolytic streptococcus.

*Treatment With 1.0 Gm. of Sulfanilamide Per Day.*—Two series were used in this group. In the first of these, series II, one of the controls and one of the treated animals recovered. The control was definitely infected as judged by temperature and lesion. In the other rabbit, infection was apparently localized since the blood was sterile and the temperature became normal after two days of treatment. The blood level was high on the first day of treatment, 18.2 mg. per cent, but dropped to about 4 mg. per cent, a level lower than in the preceding series in which only half as much drug was administered. Conjugation and elimination were comparable to what was found in the preceding series. The amount of the conjugated drug in the blood was above 5 mg. per cent in only the first specimen. The death in the other control was no doubt due to the infection, as indicated by the temperature, the nature of the lesion, and the detection of streptococci in the lesion and in a liver abscess. The other treated animal died after two days of treatment. Streptococci were found in the lesion but not in the blood. However, it is probable that the progressive accumulation of the drug in the blood, rising to



about 32 mg. per cent on the second day, had a severely toxic effect on the animal and probably was a contributory factor in causing death. The amount of conjugated drug in the blood was only about 5 per cent of the total, a very low conjugation, but in the urine it rose to an average of 25 per cent of the total.

In the other series of this group, series III, all the animals recovered. The two treated animals, rabbits 41 and 42, recovered after treatment over a period of three weeks.

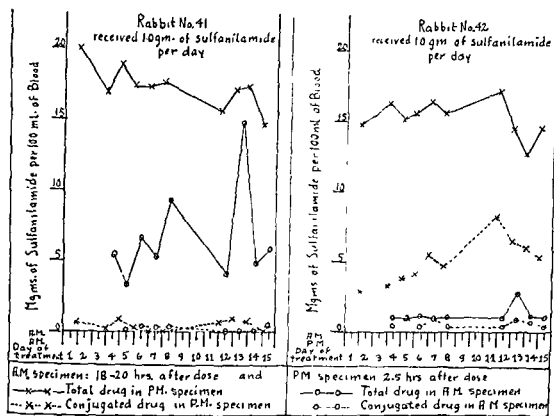


Fig. 4.—Maximum and minimum blood levels of total and conjugated sulfanilamide in rabbits infected with beta hemolytic streptococcus.

Blood and urine data are given for the first period of treatment. Table IV and Fig. 4 present the complete picture for the drug in the blood during the first two weeks of treatment. Rabbit 42 recovered more quickly than rabbit 41, although both maximum and minimum blood levels were lower in the former. The conjugation was strikingly different, uniformly low in rabbit 41, scarcely rising above 5 per cent of the total at any time, and uniformly much higher in rabbit 42. Conversely, the amount of free drug in the blood in rabbit 41 were uniformly high and relatively much greater than in rabbit 42. Whether this relatively higher level of free drug in rabbit 41 had any bearing on its slower recovery and manifestation of symptoms of toxic poisoning, including a temporary anemia, is uncertain. At any rate, this should be borne in mind along with the contention that free sulfanilamide is therapeutically more effective and that the conjugated drug is more toxic.

Elimination data are strikingly similar. There was an initial lag in recovery of the intake, then a rise up to a fairly even rate. During seventy-two hours of elimination subsequent to complete cessation of intake, almost identical amounts were recovered in the urine. This appears to represent the approximate amount of drug retained in the body during continuous treatment at the given dosage of the drug. Finally, a higher percentage of conjugated

TABLE IV

SULFANILAMIDE IN BLOOD AND URINE OF RABBITS: STREPTOCOCCUS INFECTION  
(Intake of drug 1.0 Gm. per day = approximately 0.5 Gm. per kg. of body weight.)

DAYS TREATED	TIME OF BLOOD SPEC- IMEN	RABBIT 41 (RECOVERED)					RABBIT 42 (RECOVERED)					
		SULFANILAMIDE					SULFANILAMIDE					
		IN BLOOD		IN URINE			IN BLOOD		IN URINE			
		TOTAL MG. PER 100 ML.	PER CENT CONJU- GATED	COL- LEC- TION PE- RIOD HOURS	RECOV- ERY OF INTAKE PER CENT	PER CENT CONJU- GATED	TOTAL MG. PER 100 ML.	PER CENT CONJU- GATED	COL- LEC- TION PE- RIOD HOURS	RECOV- ERY OF INTAKE PER CENT	PER CENT CONJU- GATED	
1	A.M.*											
2	P.M.	16.95	1.1	24	48.5	17.5	16.4	19.8	24	47.1	53.6	
	A.M.	5.20	-				1.04	44.3				
	P.M.	18.8	4.3	24	47.5	21.5	15.15	25.4	24	76.7	58.6	
3	A.M.	3.28	1.8				0.97	100.0				
	P.M.	17.4	1.7	24	74.3	15.9	15.6	26.6	24	69.9	60.4	
4	A.M.	6.67	6.3				1.13	33.6				
	P.M.	17.3	0.0	24	62.3	19.1	16.5	34.2	24	56.5	57.0	
5	A.M.	5.21	5.6				0.95	100.0				
	P.M.	16.95	0.0	24	59.2	16.3	15.56	30.6	24	59.3	56.8	
6	A.M.	9.25	3.4				1.0	40.0				
7 & 8	No treatment on 6th, 7th, and 8th days			72	84.6	20.7	No treatment on 6th, 7th, and 8th days			72	83.5	58.4
9	A.M.	-	-				-	-				
	P.M.	15.6	3.9	24	55.1	19.0	17.23	48.2	24	44.8	58.0	

\*A.M. = morning specimen, eighteen to twenty hours after last dose (minimum blood level).

P.M. = evening specimen, two and one-half hours after last dose (maximum blood level).

TABLE V

SULFANILAMIDE IN BLOOD AND URINE OF RABBITS: STREPTOCOCCUS INFECTION  
(Intake of drug 2.0 Gm. per day = approximately 1.0 Gm. per kg. of body weight.)

DAYS TREATED	TIME OF BLOOD SPECI- MEN	RABBIT 10 (DIED, DRUG DEATH?)					RABBIT 30 (DIED, DRUG DEATH?)				
		SULFANILAMIDE					SULFANILAMIDE				
		IN BLOOD		IN URINE			IN BLOOD		IN URINE		
		TOTAL MG. PER 100 ML.	PER CENT CONJU- GATED	COL- LEC- TION PE- RIOD HOURS	RECOV- ERY OF INTAKE PER CENT	PER CENT CONJU- GATED	TOTAL MG. PER 100 ML.	PER CENT CONJU- GATED	COL- LEC- TION PE- RIOD HOURS	RECOV- ERY OF INTAKE PER CENT	PER CENT CONJU- GATED
1	A.M.*	-	-	-	-	-	-	-	-	-	-
	P.M.	-	-	-	-	-	22.5	30.0	24	24.4	35.6
2	A.M.	3.72	100.0				5.04	83.8			
	P.M.	19.11	27.8	24	38.8	64.1	31.2	36.5	24	1.1	51.0
3	A.M.	5.71	77.2				9.4	84.0			
	P.M.	26.15	33.4	24	19.0	62.0	30.3	37.2	24	0.9	68.0
4	A.M.	22.0	95.1				16.8	86.6			
	P.M.	24.6	56.5	24	15.8	61.2	48.6	27.1			
5	A.M.	13.5	69.2				No treatment on 5th day				
	P.M.	37.9	-	24	30.8	72.7			48	93.2	52.0
6	A.M.	33.7	76.9				Died two days later apparently from toxic effect of the drug				
No treatment after 5th day; died two days later											

\*A.M. = specimen eighteen to twenty hours after last dose in case of both rabbits.

P.M. = specimen four and one-half hours after last dose for rabbit 10 and two and one-half hours after last dose for rabbit 30.

drug in the urine than in the blood reflects the greater ease of the elimination of the conjugated form, as previously indicated.

*Treatment With 2 Gm. of Sulfanilamide Per Day.*—Four series of rabbits were given this treatment. In series IV, the first of this group, both controls recovered. One of the treated animals, rabbit 10, died after five days of treatment. Death was apparently due to the effect of the drug, which was poorly eliminated and accumulated in the blood (Table V), gradually rising to a level of 33.7 mg. per cent. This appears to indicate an injury to the eliminating mechanism, possibly the kidneys. It should be noted that the amount of conjugated drug in the blood was continuously high after the second day of treatment, rising to above 25 mg. per cent. Conjugation, on the other hand, seems to be in accord with the usual finding in rabbits, indicating normal functioning of the liver at least so far as this conjugating mechanism is involved. The other treated rabbit showed a tendency to accumulation of drug in the blood. No urine studies were made. Death was due to an accident during treatment.

In series V, the second of this group, both controls died three weeks after inoculation. Hemolytic streptococci were cultured from the local lesions at autopsy. One of the treated animals died after one day of treatment. The infection was extremely acute, as evidenced by a temperature of 106° F. Hemolytic streptococci were cultured from the local lesion and from the peritoneal exudate. The other animal recovered after intermittent treatment over a period of five weeks. The characteristic picture in this animal consisted of flare-ups of infection after periodic discontinuance of the drug. This phenomenon was observed in other series. Recovery from the infection was complete at the end of the experiment. The drug was well handled, as judged by blood levels and elimination in the urine. Toward the end of the experiment a tendency toward accumulation of the drug in the blood was noted, along with lowered conjugation, which suggests injury to the mechanism of elimination and conjugating power of the liver. The maximum blood levels ranged around 20 to 25 mg. per cent. It may be that the high level during the last period of treatment, about 30 mg. per cent for the maximum values, was responsible for the final clearing up of the infection.

In series VI, the third of this group, one of the controls died of the infection. The other control recovered. In one of the animals, rabbit 24, treatment was continued over five weeks due to recurrent flare-ups of the infection. The drug was handled well during the first periods of treatment. Elimination and conjugation, as judged by blood and urine values, were apparently normal at first, but toward the end of the experiment there was, as in rabbit 25, a tendency to accumulation of drug in the blood and a definitely lowered tendency to conjugation. The maximum blood level values ranged around 20 mg. per cent during the first several periods of treatment, but thereafter, due to the accumulation in the blood, they rose at times to well above 30 mg. per cent. The temperature was subnormal for five days prior to death, and at autopsy no organisms could be demonstrated in the blood or from the site of injection. Death was undoubtedly caused by the drug.

The other animal died after treatment over a period of ten days. There was an accumulation of the drug in the blood which was manifest from the beginning. The amount of the conjugated form was high throughout, rising to a maximum of 33.8 mg. per cent. Elimination was normal at first, but decreased during the last two days. Although hemolytic streptococci were demonstrated in the local lesion at autopsy, we feel that death was primarily due to the drug.

In the final series of this group both controls died, one of them on the second day after inoculation. Autopsy revealed multiple coccidial abscesses in the liver, as well as hemolytic streptococci in the local lesion. The other control died of streptococcic infection in four days. One of the treated animals, rabbit 29, recovered (Fig. 3), but treatment had to be continued over two weeks, due to two flare-ups of infection. Maximum blood levels were high, repeatedly well above 40 mg. per cent. Conjugation was consistently low, so that the amount of conjugated drug in the blood rarely exceeded 10 mg. per cent, notwithstanding the high values for total drug. Elimination was low, less than half of the intake, which probably accounts for the high blood values. It is of particular interest to contrast the blood levels of rabbits 29 and 37, high in one and low in the other (Fig. 3). They responded to treatment in about the same manner. Both had to be treated repeatedly after recurrent flare-ups of infection. The amount of conjugated drug was consistently low over the entire period of treatment and neither manifested any toxic effects.

The other treated animal, rabbit 30, probably died from the effect of the drug. The blood level rose gradually to almost 50 mg. per cent. Elimination was poor and for two days there was an almost complete anuria.

Thus, in this group (series IV to VII), 5 of the controls died of the infection. Of the treated animals, 3 recovered from the infection after repeated flare-ups, but one of these died from the toxic effects of the drug. All of them manifested a definite tendency to drug retention toward the end of the treatment. The death of the other 4 treated animals may be ascribed to the effect of the drug, associated with an accumulation of the drug in the blood, a lowered elimination, and a definite anuria in at least one of these.

#### SUMMARY AND CONCLUSIONS

1. Blood and urine studies were made on the levels and distribution of sulfanilamide in seven series of rabbits infected with hemolytic streptococci.

2. All the animals received similar inoculations, but marked individual differences of susceptibility to the infective organism were noted.

3. Recurrent flare-ups of infection were observed in a number of animals. Complete clinical cure required resumption of treatment at varying intervals.

4. A definite tendency toward accumulation of the drug in the blood and poor elimination was noted in the majority of the treated animals and was a contributory cause of death in at least 5. In all the animals in which the drug was a definite factor in causing death, the level of the conjugated form was relatively high.

5. Most favorable results in the treatment of infection were obtained with the highest dosage used, approximately 1 Gm. per kilogram of body weight, with a drug level ranging between 20 and 30 mg. per cent.

6. For a given intake of the drug the elimination in the urine reaches a maximum level within two or three days after the beginning of treatment. The conjugated drug is more readily eliminated than free sulfanilamide, irrespective of the level in the blood.

We wish to express our appreciation to Professors Randle C. Rosenberger and George R. Bancroft whose interest has made it possible to carry out this investigation.

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## THE CALORIGENIC ACTION OF AMMONIUM SALTS IN THE HUMAN SUBJECT\*

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GRAFE<sup>1</sup> reported his studies of the specific dynamic action of protein in rabbits, dogs, and men. He considered ammonium carbonate, ammonium chloride, acetamide, and asparagine per gram of nitrogen comparable to glycine and alanine in increasing heat production. He concluded that the nitrogen of amino acids was the principal factor in their specific dynamic action and the combustible fraction was secondary. Of the substances he tested, ammonium chloride had a much greater calorigenic effect than the amino acids. Lundsgaard<sup>2</sup> compared the calorigenic action of sodium and ammonium glycocollate to that of glycine and that of sodium and ammonium lactate to that of alanine in normal conscious dogs. He reported that the sodium salts had slight effect, while that of the ammonium salts was very similar to that of the corresponding amino acids. This report is an extension of Lundsgaard's studies in the human subject.

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TABLE I

EFFECT OF AMINO ACIDS AND AMMONIUM AND SODIUM SALTS OF THE CORRESPONDING ACIDS UPON OXYGEN CONSUMPTION

SUB- JECT	TEST SUBSTANCE	OXYGEN CONSUMPTION						NUMBER OF TRIALS
		BASAL C.C. PER MIN.	PER CENT INCREASE ABOVE BASAL					
			MINUTES AFTER INGESTION OF DRUG				AVERAGE OF MAXIMUM INCREASE OF ALL TRIALS	
			30	60	90	120		
C	Alanine	220	12.6	15.7	5.2	2.1	17.8	2
	Alanine	200	15.5	20.2	-	15.5		
	Ammonium lactate	209	3.1	11.1	20.0	11.1	20.5	5
	Ammonium lactate	209	13.0	21.7	13.0	0		
	Sodium lactate	208	8.4	8.4	3.0	-	10.1	6
	Sodium lactate	218	1.2	1.2	6.3	7.6		
	Repeated basals	202	2.8	0	0	5.6		3
N	Alanine	214	25.3	-	34.0	9.1	22.5	2
	Alanine	212	11.1	11.1	8.4	8.4		
	Ammonium lactate	220	8.2	-	13.4	5.6	13.6	5
	Ammonium lactate	220	7.5	18.0	18.0	13.0		
	Sodium lactate	209	5.7	5.7	5.7	8.4	6.7	5
	Sodium lactate	212	9.0	3.1	5.8	3.1		
	Repeated basals	213	0	0	0	5.7		3
J	Alanine	236	1.2	5.2	0	1.2	6.5	
	Alanine	228	-	11.6	7.4	7.4		
	Ammonium lactate	237	17.1	16.0	19.9	9.7	18.2	
	Ammonium lactate	236	20.9	13.8	11.0	13.8		
	Sodium lactate	228	0	5.7	12.2	9.6	11.1	
	Sodium lactate	244	9.5	6.8	4.5	0		3
	Repeated basals	229	0	2.5	2.5	0		
R	Glycine	216	8.7	12.0	10.1	-	12.6	2
	Glycine	203	13.3	13.3	12.3	-		
	Ammonium acetate	212	11.2	11.2	11.2	12.5	7.9	4
	Ammonium acetate	211	7.5	7.5	-	-		
	Sodium acetate	221	8.5	4.5	-	-	5.0	4
	Sodium acetate	233	0	0	-4.2	-		
S	Glycine	222	8.1	8.1	8.1		10.9	2
	Glycine	211	4.7	13.7	9.0			
	Ammonium acetate	226	16.3	-	-		14.6	3
	Ammonium acetate	241	2.1	17.4	9.9			
	Sodium acetate	213	8.9	8.9	12.2		11.4	2
	Sodium acetate	227	0	4.5	-			
Mc	Glycine	195	8.9	3.5	3.5		6.0	2
	Ammonium acetate	200	9.9	19.8	23.4		16.3	5
	Ammonium acetate	181	16.0	19.9	8.8			
	Sodium acetate	196	5.1	5.9	0			
	Sodium acetate	197	0	0	5.1		5.4	3

## EXPERIMENTAL PROCEDURE

The oxygen consumption of 6 normal medical students was measured by a Benedict-Sanborn metabolism machine. The subjects were accustomed to breathing through the apparatus before data collection began. The tests were made in the morning at least fifteen hours after the last meal and after a thirty-minute rest upon a cot. The basal oxygen consumption was ascertained, and the test substance was administered orally in 75 c.c. of water in a dosage of 0.133 molar. The oxygen consumption was then measured thirty, sixty, ninety, and one hundred and twenty minutes after ingestion of the test substance. The alanine was a racemic mixture. The basal oxygen values were corrected for temperature and barometric pressure.

Control observations were made by the ingestion of 75 c.c. of water, an isomolar amount of sodium bicarbonate or sodium chloride, or repeated basals without the ingestion of any substance. The psychic reactions to the administration of these substances were very slight.

### RESULTS

To conserve space only representative data are presented in Table I, together with the average maximum increase for each substance. The maximum variation in the controls with water and repeated basals never exceeded 4 per cent. The increases effected by the sodium salts, lactate, acetate, bicarbonate, and chloride were about the same, ranging from 5.7 to 10.7 per cent. On the other hand, the average calorigenic effect of ammonium lactate was 89 per cent greater than that of sodium lactate and that of ammonium acetate was 138 per cent greater than that of sodium acetate. The ammonium salts were more effective than the corresponding amino acids. For the most part, the response to the effective drugs came at sixty to ninety minutes, and to the sodium salts at thirty minutes. The length of the latent period rules out any psychic effects of taking the drugs. The duration of the response was greatest for alanine, and the ammonium salts, being 120 minutes or more. For the sodium salts, the effect seldom lasted longer than eighty minutes. The urine output in the two hours after taking the salts was always less than after the amino acids.

### DISCUSSION

The data indicate that ammonia exerts a definite calorigenic effect in the human body. It might well be considered as additional evidence supporting the idea advanced by others<sup>3</sup> that the ammonia arising from the deamination of amino acids is largely responsible for the specific dynamic action of protein. In our experiments the sodium salts of acetic and lactic acids had no greater effect than the bicarbonate or chloride. This might indicate that the residue after deamination has no appreciable specific calorigenic action and that the effect of all the sodium salts in these experiments could be ascribed to osmotic pressure changes. The shorter latent period for maximum response to the sodium salts than for the ammonium salts supports this idea.

The mechanism by which ammonia exerts a calorigenic action is unknown, but it has been demonstrated to exert effects upon the metabolism of tissues *in vitro*. Annau<sup>4</sup> and Edson<sup>5</sup> reported that ammonia increased the rate of acetoacetic acid formation in liver slices. Edson found that ammonium chloride increased the respiration of kidney tissue, had no effect upon brain or spleen, and slightly inhibited the striated muscle. Krebs<sup>6</sup> has reported that in the presence of glutamic acid, kidney and nervous tissue take up large quantities of ammonia to form glutamine. In the brain and retina the quantity of ammonia disappearing exceeded the amount of amide nitrogen formed. Neber<sup>7</sup> has shown that surviving rat liver forms large quantities of amino nitrogen when ammonium pyruvate is added. Since the formation of the amino acids proceeds in the absence of oxygen, the reaction must be exothermic. Krebs and Henseleit<sup>8</sup> reported that urea formation from ammonia in liver slices re-

quired tissue respiration. Borsook and Jeffreys,<sup>9</sup> after theoretical considerations, decided that urea formation from ammonia could not occur spontaneously but must depend upon an energy-yielding reaction. Some of these reactions or effects might have occurred in our experiments and caused either directly or indirectly increased heat production.

The responses of subject J to the ingestion of alanine were only 2.4 and 5.2 per cent increase, but when the amount of alanine was doubled, the per cent increase was 11.8. About six months after these experiments, the subject's physical complaints led to the diagnosis of a moderate degree of diabetes mellitus. In this connection it is interesting to note that Grafe found that the administration of 50 Gm. of glycine to a depancreatized dog resulted in a 10 per cent decrease in oxygen consumption and carbon dioxide production.

#### SUMMARY

In 3 normal subjects, with a dosage of 0.133 molar, the ingestion of alanine produced an average increase in oxygen consumption above the basal value of 14.6 per cent; ammonium lactate 17.4 per cent and sodium lactate 9.2 per cent. In 3 other human subjects the ingestion of glycine produced an average increase in oxygen consumption above the basal level of 9.8 per cent, ammonium acetate 13.6 per cent, and sodium acetate 5.7 per cent. Isomolar doses of sodium bicarbonate and sodium chloride caused oxygen consumption to be increased on the average of 10.7 and 9.9, respectively.

The comparable calorogenic effects of these ammonium salts to the corresponding amino acids, alanine, and glycine are in agreement with the studies of Grafe and Lundsgaard. The marked difference in the calorogenic effects of the ammonium and sodium salts might be interpreted to mean that the specific dynamic action of amino acids is due to their amino group and not to their non-nitrogenous fraction. The similarity of the calorogenic effects of the sodium salts of either organic or inorganic acids, and the shorter latent period for maximum response, might be attributed to osmotic effects rather than to oxidative processes.

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# THE TOXIC EFFECTS OF SULFANILAMIDE UPON TISSUES OF RATS\*

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THE introduction into therapeutics of prontosil I by Domagk<sup>1</sup> in 1935 was followed by widespread clinical and experimental investigations which have confirmed Domagk's original observations. In 1935 Tréfouël, Nitti, and Bovet<sup>2</sup> came to the conclusion that it was the sulfonamide grouping which was responsible for the therapeutic activity of prontosil I. Their work was confirmed in 1936 by Goissedet, Despois, Gailliot, and Mayer,<sup>3</sup> Buttle, Gray, and Stephenson,<sup>4</sup> Gley and Girard,<sup>5</sup> and Colebrook and his co-workers.<sup>6</sup> The reduction of prontosil I and II was accomplished by Bliss and Long<sup>7</sup> in 1937. Shortly thereafter, it was demonstrated (Fuller<sup>8</sup>) that the oral or parenteral administration of prontosil I and II or of sulfanilamide was followed by the appearance of free sulfanilamide and acetyl sulfanilamide in equal proportions in blood and urine. Considerable success has attended the use of these compounds in streptococcal, meningococcal, and gonococcal infections in human beings. Much speculation has arisen regarding the mechanism of the therapeutic activity and, in Table I, is presented a brief summary of the hypotheses which have been put forward by various workers.

TABLE I  
VIEWS CONCERNING MODE OF ACTION OF SULFANILAMIDE

ACTION UPON MICROORGANISM	ACTION UPON HOST
1. Bacteriostasis (Colebrook et al.; Bliss and Long)	1. Activation of phagocytic activity of cells (Domagk)
2. Inhibition of formation of hemolysins and negatively chemotactic substances (Levaditi and Vaisman; Meyer; Osgood and Brownlee)	2. Stimulation of reticulo-endothelial system (Burgers; Gley and Girard; Levaditi and Vaisman)
3. Interference with capsule formation or capsule destruction (Levaditi; Lyons)	3. Combination of sulfanilamide with body protein rendering it unfit medium for microorganisms (Levaditi)
4. Interference with protein-digesting enzymes (Lockwood)	
5. Bactericidal (Frankl)	

It has become apparent from widely scattered experimental and clinical reports that sulfanilamide and related compounds possess a definite toxicity both for man and for the lower animals. The toxic dosage in different species

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of animals, and even in different individuals of the same species, varies. The present investigation has been undertaken to determine the pathologic changes in the tissues of rats following prolonged and excessive administration of sulfanilamide.

#### TOXICITY OF SULFANILAMIDE

Much uncertainty still exists regarding the maximum tolerated dose and the minimum lethal dose of sulfanilamide in various animals. The tolerated amount in mice has been placed at 2.5 Gm. per kilogram (Buttle, Gray, and Stephenson<sup>4</sup>), 0.9 Gm. per kilogram (Long and Bliss<sup>9</sup>), 2 Gm. per kilogram (Raiziss, Severac, and Moetsch<sup>10</sup>), 2.5 Gm. per kilogram (Whitby<sup>11</sup>), and 3 to 4 Gm. per kilogram (Rosenthal<sup>12</sup>). In rabbits the maximum tolerated dose has been found to be 1 Gm. per kilogram (Tréfouël and co-workers<sup>2</sup>), 5 Gm. per kilogram (Nitti and Bovet<sup>14</sup>). The fatal dose in mice varies from 3 Gm. per kilogram (Raiziss, Severac, and Moetsch<sup>10</sup>) to 7.5 Gm. per kilogram (Whitby<sup>11</sup>), and 10 Gm. per kilogram (Buttle, Gray, and Stephenson<sup>4</sup>).

TABLE II  
SUMMARY OF TOXIC EFFECTS OF SULFANILAMIDE

HUMAN BEINGS	LOWER ANIMALS
1. Nausea	1. Spasticity of extremities
2. Vomiting	2. Excitability
3. Dizziness	3. Vestibular dysfunction
4. Malaise	4. Ataxia
5. Headache	5. Diminished reflex, excitability
6. Dermatitis	6. Motor paralysis
1. Angioneurotic edema	7. Partial narcosis
2. Maculopapular	8. Hypothermia
3. Morbilliform	9. Cardiac depression
7. Fever	10. Fall of blood pressure
8. Acidosis	11. Loss of weight
9. Leucocytosis	
10. Hemolytic anemia	
11. Agranulocytosis	
12. Methemoglobinemia	
13. Sulfhemoglobinemia	
14. Jaundice with decrease in liver function	
15. Acute yellow atrophy of liver	
16. Peripheral and optic neuritis	
17. Nephritis	

The toxic symptoms in these animals varied (Table II): spasticity of the extremities, excitability, incoordination, vestibular dysfunction, ataxia, somnolence, lessened reflex excitability, motor paralysis, deepened respiration, and partial narcosis. A reduction of the body temperature and of the respiratory quotient has been noted in rats. Large intravenous doses produce a fall in blood pressure which is associated with a direct depressant action upon the cardiac muscle and a dilatation of the visceral blood vessels. These toxic effects are of an acute nature and do not resemble the symptoms of toxicity seen in human beings.

Various toxic manifestations have been noted in human beings in response to the administration of sulfanilamide (Table II). In a series of 335 patients Long, Bliss, and Feinstone<sup>15</sup> observed dizziness, headache, loss of ability to

concentrate, nausea, and vomiting in many. A considerable number of these patients demonstrated the presence of cyanosis with or without methemoglobinemia and sulfhemoglobinemia. Acidosis associated with hyperpnea and a lowered carbon dioxide combining power of the blood occurred in 3 per cent of the patients. Jaundice accompanied by a diminution of liver function was present in one. Skin rashes were noted in 1 per cent of the patients and fever in 6 per cent. They found that a chronic hemolytic anemia developed in a considerable number, while acute hemolytic anemia was seen in 3 per cent of their series. Agranulocytosis was found in one patient. Fatal agranulocytosis has been reported by several observers (Young<sup>16</sup> Plumer,<sup>17</sup> Berg and Holtzman,<sup>18</sup> O'Connell,<sup>19</sup> Schwartz and co-workers,<sup>20</sup> Sheeket and Price,<sup>21</sup> and others). Death from acute yellow atrophy of the liver, as a result of the administration of sulfanilamide, has been reported (Cline<sup>22</sup>). Peripheral neuritis (Ornstein and Furst<sup>23</sup>) and optic neuritis (Bucy<sup>24</sup>) have been included among the toxic manifestations.

#### METHODS

A series of 80 white rats, each weighing from 200 to 250 Gm., were used in this investigation. Of these, 40 served as controls and the remainder were divided into 4 groups of 10 rats to each group. The animals were observed for a period of two weeks before being used, and all unhealthy ones were discarded. An adequate amount of water and food was provided. Sulfanilamide,\* in a 10 per cent suspension in sterile doubly distilled water, was injected beneath the skin of the anterior abdominal wall daily over a period of three months. All equipment used was sterilized before each injection, and every effort was made to maintain a reasonable degree of sterility. The following dosages of sulfanilamide were used: B group received 0.5 Gm. per kilogram of body weight; C group received 1.0 Gm. per kilogram; D group received 1.5 Gm. per kilogram; E group received 2.0 Gm. per kilogram. At intervals of ten days from the commencement of the experiment, one animal from each group was killed by a blow upon the head. The tissues were immediately removed and placed in a 10 per cent formalin solution, or in absolute alcohol (where glycogen stains were to be made). The tissues were embedded in celloidin, and the following stains were used: hematoxylin-eosin, Best's carmine stain for glycogen, Mallory's stain for iron, scarlet red stain for fat.

#### RESULTS

Shortly after the injections were made the animals exhibited a marked disinclination to movement and remained stationary in one corner of the cage. Some of the rats, however, appeared to be unaffected by the sulfanilamide and remained active. Many showed a considerable degree of thirst during the first few days of injection. No exact data were obtainable concerning the amount of water intake and output, but our observations indicated an increased water intake during the earliest periods of the administration of the drug. By the end of the first week, however, a normal level of water intake had been at-

\*We are indebted to the Medical Research Division, Eli Lilly Co., Indianapolis, Ind., for kindly supplying us with the sulfanilamide used in this investigation.



Fig. 1.—A, Subcutaneous tissue of anterior abdominal wall ( $\times 175$ ). Foreign body reaction with giant cell formation. B, The same specimen ( $\times 625$ ). Sulfanilamide crystal lying within giant cell. C, Rat: necrosis of skin of anterior abdominal wall.

tained. Some of the animals revealed a considerable degree of cyanosis of the mucous membranes of the mouth and tongue, which apparently was not related definitely to the amount of sulfanilamide given. It soon became apparent that the greatest reaction to the sulfanilamide was exhibited during the first few days of the injection and, if the animal could withstand the toxic action during this period, it was likely to survive indefinitely. It was found to be possible to increase the tolerance to the drug gradually, so that in some animals as much as 6 Gm. per kilogram were injected without harmful results ensuing. The mechanism of this induced tolerance is unknown. Another factor, which became evident, was the individual idiosyncrasy of these animals to

sulfanilamide. Some exhibited a reaction to the smallest amount given and others were apparently unaffected even by large amounts. Most of the animals showed a moderate weight loss which became accentuated when, and if, necrosis occurred at the site of injection.

*Pathology at Injection Site.*—The subcutaneous deposition of the amorphous crystals of sulfanilamide resulted in changes, both in the crystals and in the surrounding tissue. After a few days the crystals began to disintegrate into smaller needle-shaped particles and, finally, into a granular appearing mass in which all traces of a crystalline structure had disappeared. Complete disappearance of the injected material took place relatively slowly, and traces were still present as long as two months after the final injection. The first local response to the introduction of the sulfanilamide into the tissue was a marked infiltration by inflammatory cells, most of which were wandering mononuclear cells and lymphocytes. This was followed by proliferation of the local histiocytes and fibrous connective tissue cells. Numerous giant cells of the Langhans type formed, and in many of these could be seen crystalline and granular material. The proliferating fibroblasts showed the presence of frequent mitotic figures (Fig. 1, A and B). A moderate degree of tissue eosinophilia was also evident. This foreign body response in the tissues varied in each animal quantitatively but not qualitatively. It was most apparent during the earlier periods of injection of sulfanilamide. After a period of two months examination of the injection site revealed the presence of adult fibrous connective tissue showing few mitotic figures. A moderate number of mononuclear cells was scattered throughout the tissue. Giant cells were absent or few in number. Moreover, practically all the injected sulfanilamide crystals had been removed. Necrosis occurred at the site of injection in several animals and was usually associated with a moderate grade of infection (Fig. 1, C).

TABLE III  
BLOOD PICTURE IN CONTROL RATS

GROUP	RED BLOOD CELLS	HEMOGLOBIN GM.	WHITE BLOOD CELLS
A-3	6,250,000	14.8	2,000
A-4	5,550,000	16.8	4,400
A-5	6,700,000	16.6	3,520
A-6	6,400,000	16.2	3,950
A-7	5,250,000	13.6	2,900
A-8	6,100,000	16.1	4,500
A-9	5,250,000	13.5	2,300
A-10	5,600,000	12.8	3,100
Average	5,887,500	15.03	3,333

*Blood Picture.*—Complete blood studies were made in many of the control series and in most of the sulfanilamized rats. Blood was obtained in both series of animals in an exactly similar manner, by killing the rats and taking blood directly from the heart. It will be noted (Table III) that representative data indicate that, in normal rats, the erythrocytes vary from 5,250,000 per cm. to 6,700,000 per cm., with an average count of 5,887,500 per cm. The hemoglobin varied from 12.8 Gm. to 16.8 Gm. per 100 c.c. of blood, with an average of 15.03 Gm. per cent. The leucocyte count varied from 2,000 per

cm. to 4,500 per cm., with an average count of 3,333. The sulfanilamized rats revealed a definite reduction of both the red blood cells and the hemoglobin. This reduction was directly proportional to the amount of sulfanilamide given (Table IV). However, the factor of individual idiosyncrasy to the drug was present, but was evidently minor to that of dosage. In some of these animals the anemia was quite severe. The mechanism of this anemia must be subjected to examination. Two possibilities may be considered: (1) a direct action of sulfanilamide upon the erythropoietic elements in the bone marrow; (2) a direct hemolytic action of the drug upon the circulating erythrocytes. Evidence will be presented later in this paper which supports the view that this anemia is hemolytic in origin. Representative data (Table IV) regarding the leucocyte count reveal several interesting facts. While the average count indicates a mild leucocytosis, a further analysis discloses the fact that with B group of rats (0.5 Gm. per kilogram) the count is unaffected.

TABLE IV  
INFLUENCE OF SULFANILAMIDE UPON BLOOD OF RATS

GROUP	RED BLOOD CELLS	HEMOGLOBIN GM.	WHITE BLOOD CELLS
B-1	4,500,000	13.8	3,200
B-2	3,700,000	13.2	3,520
C-2	5,200,000	13.2	5,200
C-4	5,350,000	14.8	8,000
D-2	4,800,000	12.0	5,400
D-3	3,700,000	10.2	3,100
D-4	4,950,000	13.1	6,400
D-5	5,350,000	15.0	6,400
E-2	2,300,000	11.0	3,400
E-5	3,900,000	12.0	3,100
Average	4,375,000	12.83	4,772

C group (1.0 Gm. per kilogram) shows a definite leucocytosis, as does also group D (1.5 Gm. per kilogram). However, in group E (2.0 Gm. per kilogram) the count has returned to normal or slightly below normal. Apparently, dosage is important, inasmuch as moderate amounts of sulfanilamide cause leucocytosis, while large amounts return the count to normal or lower. The tentative conclusion might be drawn that sulfanilamide affects the red blood cells more readily than the white blood cells and that the earliest evidence of toxicity is a leucocytosis and later a leucopenia.

*Bone Marrow.*—The bone marrow was obtained by sectioning the left femur at the junction of the middle and upper thirds. The changes found were slight in most animals. A mild hyperplasia of the myeloid elements of the bone marrow and an increase in the number of megakaryocytes (Fig. 2, A) occurred. No other significant alterations were present. However, in one rat of the sulfanilamized series the bone marrow revealed a definite arrest of maturation of the myeloid cells (Fig. 2, B). These cells were seen to consist mainly of myeloblasts and early myelocytes. No lobing of the nuclei could be detected, and there was a complete absence of mature polymorphonuclear leucocytes. This bone marrow also revealed a decrease in the number of megakaryocytes. The total cellularity of the marrow was not diminished.

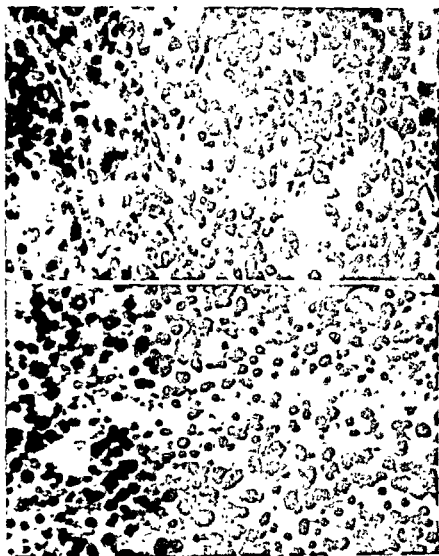


Fig. 2.—A, Femoral bone marrow ( $\times 625$ ). Hyperplasia of myeloid elements. B, Femoral bone marrow ( $\times 625$ ). Arrest of maturation of myeloid cells

*Spleen.*—The spleen exhibited several interesting changes. In a number of animals, one or more nodular protuberances occurred upon the surface of the splenic capsule (Fig. 3, A). These consisted of a proliferation of young fibroblasts in which small amorphous crystals could be found. Occasionally, a foreign body giant cell, containing a crystal, was noted, as was also a mild infiltration by mononuclear cells and eosinophile leucocytes (Fig. 3, B). The mechanism of the crystal deposition will receive comment later. Examination of the splenic pulp revealed a hyperplasia of the reticulum and endothelial cells of varying degree. In only 3 animals was this marked; in these the hyperplastic cells appeared to be causing some compression of the blood spaces (Fig. 3, C). In several animals areas of focal necrosis were present and appeared to favor the centers of the Malpighian bodies. In such foci the cells were swollen or disintegrating with karyolysis and karyorrhexis of the nuclei and secondary invasion by polymorphonuclear leucocytes (Fig. 3, D). In the spleens of both the control rats and the sulfanilamized rats there was present a brownish pigment, lying in the form of granules within the reticulo-endothelial cells. However, greater amounts of this pigment were found in the spleens of the sulfanilamized animals. Appropriate stains indicated that this pigment contained iron and was probably derived from the breakdown of erythrocytes. The presence of anemia and the occurrence of iron-containing pigment in the spleen point to an excessive destruction of red blood cells. It is not possible to state, from data

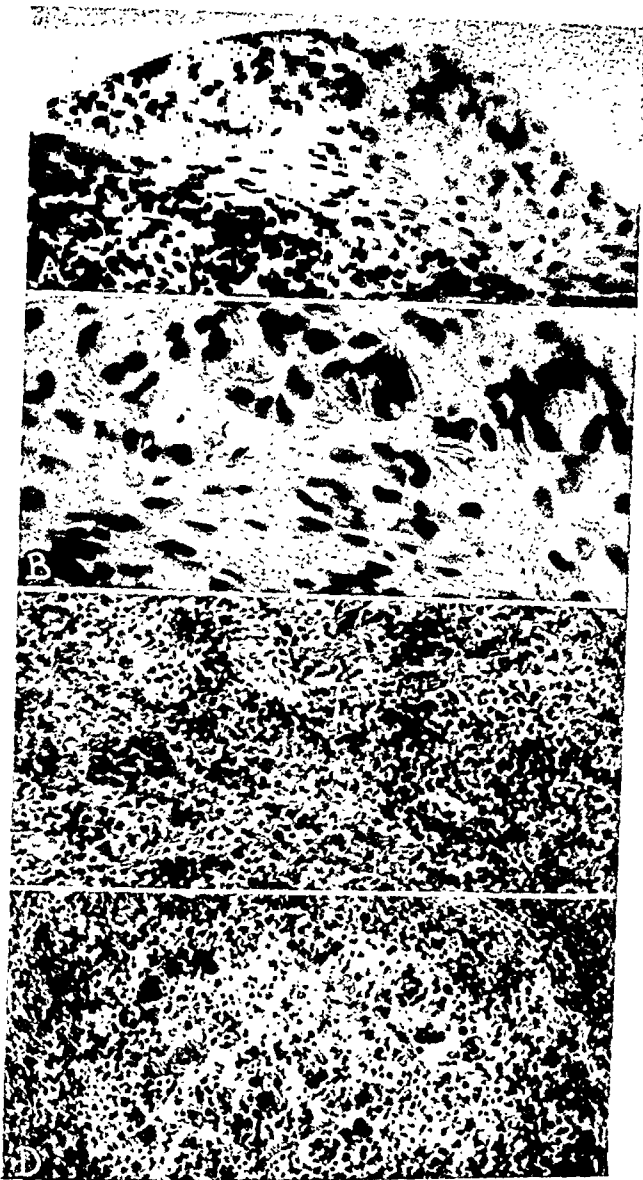


Fig. 3.—A, Capsule of spleen ( $\times 175$ ). Fibroblastic nodule with crystal deposition. B, The same specimen ( $\times 625$ ). C, Spleen ( $\times 175$ ). Hyperplasia of reticulum and endothelial cells. D, Spleen ( $\times 175$ ). Necrosis in Malpighian nodule.

obtained, whether or not this red blood cell destruction is due to a direct effect of sulfanilamide or to an excessive splenic activity in this direction. In one rat the spleen showed extramedullary hematopoiesis in the form of islands of myeloid cells. The significance of this finding is problematical.

*Liver.*—No significant changes in the size of the liver were found in the sulfanilamized animals. The liver cells revealed varying grades of alteration. The cells were enlarged, and the cytoplasm revealed the presence of numerous coarse granules (Fig. 4, A). In several animals mitoses in the liver cells were increased in number. A frequent finding was the formation of nodules of fibrous connective tissue on the outer and inner surfaces of Glisson's capsule.



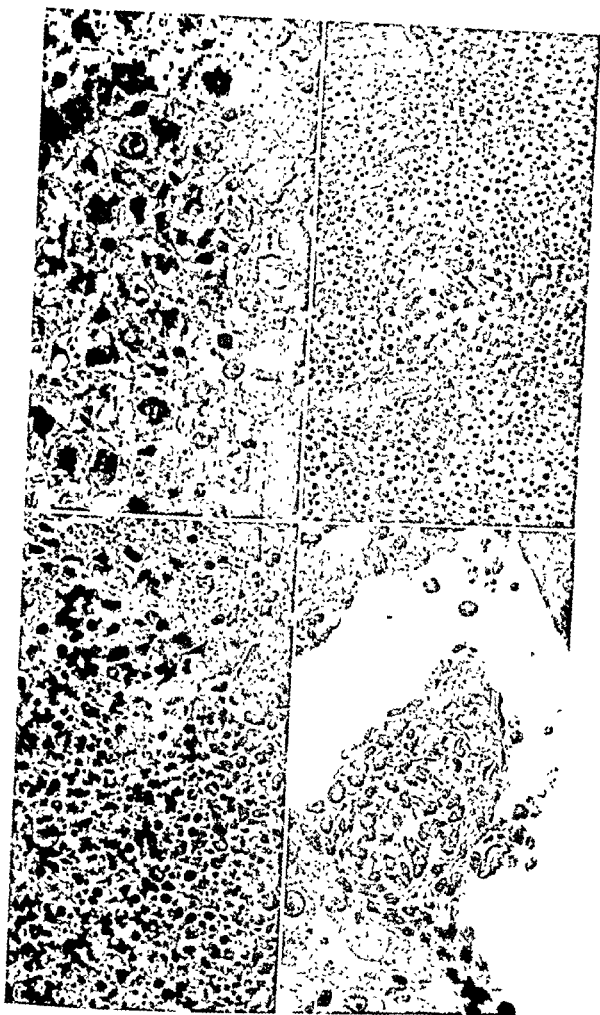


FIG. 4.—A, Liver ( $\times 625$ ). Parenchymatous degeneration of hepatic cells; occasional mitoses. B, Liver ( $\times 175$ ). Early focal necrosis. C, Liver ( $\times 625$ ). Focal necrosis with cellular exudate. D, Liver ( $\times 625$ ). Intimal nodule projecting into lumen of blood vessel; crystal deposition.

In these nodules crystal deposition could be noted. Similar formations were present within the liver and were perivascular in distribution. Occasional projections from the intima of the larger blood vessels of the liver were seen. These were composed of fibrous connective tissue in which crystals could be found (Fig. 4, *D*). In several instances irregularly placed focal areas of necrosis of the liver cells occurred. In the earliest stages the hepatic cells became pale and swollen (Fig. 4, *B*). Still later, an infiltration of polymorphonuclear leucocytes and wandering mononuclear cells took place into these areas (Fig. 4, *C*). Not infrequently, giant cells of the foreign body type could be seen surrounded by polymorphonuclear leucocytes and monocytes. No crystal deposition was noted in these focal areas of necrosis. The Kupffer cells of the liver were more prominent than usual, and many of them contained a brown pigment which was subsequently determined to be hemosiderin. Repeated fat stains were made of these livers, but only a slight increase in the amount of stainable fat was present. Glycogen stains did not reveal any abnormal changes.

TABLE V  
INCIDENCE OF TISSUE ALTERATIONS IN SULFANILAMIZED RATS

TYPE OF TISSUE CHANGE	NUMBER OF RATS PRESENTING CHANGE	PER CENT
1. Foreign body reaction at injection site	40	100
2. Secondary anemia	30	75
3. Myeloid hyperplasia in bone marrow	15	37.5
4. Myeloid arrest in bone marrow	1	2.5
5. Hemosiderosis of spleen and liver	30	75
6. Parenchymatous degeneration of liver cells	5	12.5
7. Focal necrosis of liver	4	10
8. Focal necrosis of spleen	1	2.5
9. Crystal deposition in liver, spleen, and meninges	10	25
10. Hyperplasia of reticulo-endothelial cells of spleen	6	15
11. Myelopoiesis in spleen	1	2.5

*Kidney.*—Examination of the kidneys revealed only minor alterations. A moderate degree of cloudy swelling of the epithelium of tubules, particularly that of the convoluted tubules, was noted. The glomeruli showed no significant pathology. A mild increase of fat could be detected in the tubular epithelium by the use of appropriate stains.

*Nervous System.*—In several animals there occurred microscopic proliferations of fibrous connective tissue in the meninges. In these areas deposits of small crystals, of the same type described elsewhere, were present. No other significant changes were present in the brain, spinal cord, or peripheral nerves.

*Lungs.*—A mild increase in the number of macrophage cells was noted in the sulfanilamized animals. Several rats exhibited bronchopneumonia, but this was probably an incidental finding.

*Heart.*—No significant alterations were found.

*Adrenal Gland.*—No pathology was noted.

#### COMMENT

It is evident from this study that the toxic properties of sulfanilamide manifest themselves not only in functional disturbances but also in definite

alterations of the tissues. An examination of the literature reveals a conflict of opinion regarding the tissue pathology following administration of sulfanilamide. An increased amount of fat has been noted in the epithelium of the collecting tubules of the kidneys of rats, rabbits, and dogs following sulfanilamide, but no other tissue lesions (Geiling and Cannon<sup>23</sup>). Hawking<sup>26</sup> found no lesions in the kidneys or other viscera, but reported chromatolytic changes in the neurones of the spinal cord, cerebral cortex, and midbrain. Using mice, Hageman<sup>27</sup> injected sulfanilamide both subcutaneously and intraperitoneally in a dosage of 1 to 2.5 Gm. per kilogram. He reported no evidences of visceral pathology, but noted splenic hemosiderosis and an eosinophilia in the bone marrow. The intraperitoneal injections produced a foreign body reaction in the peritoneum, with crystals of sulfanilamide scattered throughout the area of reaction. Cloudy swelling of the tubular epithelium of the kidneys and of the hepatic cells of rabbits has been observed (Kolmer, Brown, and Rule<sup>28</sup>). Nelson<sup>29</sup> has reported renal changes (degeneration of the tubular epithelium, tubular casts, and tubular dilatation), hemosiderosis of the spleen, degenerative changes in the testes and voluntary muscles, hypoplasia or hyperplasia of the bone marrow, and fatty changes in the liver. These changes occurred following the administration of fatal doses of sulfanilamide to rabbits. He also noted that the histologic changes were even more apparent in hens, in which there occurred marked fatty changes in the liver and kidney, hemosiderosis of the spleen and liver, peripheral neuritis, and hypoplasia of the bone marrow. On the contrary, other observers have been unable to find any pathologic lesions in either dogs or rats following sulfanilamide administration (Marshall, Cutting, and Emerson<sup>30</sup>). The discordance of opinion among the various workers mentioned may be due, in part, to differences of dosage, of methods of administration, of duration of administration, and finally, a variation in the type of animals used.

The mechanism of crystal deposition deserves some consideration at this point. Five possibilities present themselves: (a) Transfer of crystals from the inoculation site by phagocytes via the blood stream. (b) Accidental introduction of crystals into a subcutaneous blood vessel at the time of injection. (c) Spontaneous discharge of crystals into a blood vessel in the area of injection. (d) Accidental penetration of the peritoneal cavity at the time of injection, with deposition of crystals upon the surfaces of the liver and spleen. (e) Excessive concentration of sulfanilamide either in free or conjugated form in the blood, with subsequent crystallization into various tissues. The possibilities (a) and (d) can probably be excluded. We are unable to state, from the data available, the true explanation. Nor are we able to conclude that the crystals are those of free or conjugated sulfanilamide. Undoubtedly, however, they are related to the administration of this substance.

A further analysis of the tissue changes presented by these animals leads us to the conclusion that the activity of sulfanilamide, in this respect, can be divided into two main types: (1) a specific action, and (2) a nonspecific action. The specific action of sulfanilamide upon the recipient tissues is dependent upon its chemical structure. To this type of action can be attributed such tissue lesions as parenchymatous degeneration of hepatic and

renal epithelium, destruction of the red blood cells, and bone marrow alterations (hyperplasia, hypoplasia, and arrest of maturation of the myeloid series of cells). The nonspecific type of action is so called because we believe that it is analogous to that of any particulate substance, e.g., India ink or carbon particles. To this second type of action may be attributed the foreign body fibroblastic change at the injection site, the fibroblastic reactions at the sites of crystal deposition, the focal necrosis in the spleen and liver, and the hyperplasia of the reticulo-endothelial apparatus.

It would appear, from this study, that the factor of individual idiosyncrasy toward sulfanilamide might tend to enhance the specific rather than the nonspecific tissue effect. On the other hand, a diminution of the excretion of the substance might enhance either type of effect. Moreover, it is suggested that only the specific tissue effect would result from the oral administration of sulfanilamide, whereas the parenteral administration would be more prone to produce lesions in which both specific and nonspecific forms of activity of sulfanilamide are involved.

#### CONCLUSION

A study has been made of the effects of the prolonged administration of sulfanilamide upon the tissues of rats. It is suggested that the tissue changes resulting from sulfanilamide administration are of two types, one being dependent upon the specific chemical structure of the substance, and the other being characterized by a nonspecific foreign body response.

We wish to express our deep appreciation to Dr. J. L. Scianni, medical illustrator, University of Tennessee, College of Medicine, for the illustrations in this paper.

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## THE ACCURACY OF DIAGNOSIS OF APPENDICITIS\*

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A STUDY was undertaken to determine primarily the accuracy of diagnosis of appendicitis at the Santa Monica Hospital. By "diagnosis" we refer, first, to the preoperative or clinical diagnosis; second, to the surgical or operative diagnosis; and third, to the postoperative or laboratory diagnosis. The first two diagnoses are made by the surgeon. The last is made by the pathologist and is our standard of accuracy.

By "appendicitis" we refer to inflammatory diseases of the appendix as they are commonly understood. We are aware that there is current considerable argument to the effect that removal of the pathologically normal appendix will afford relief from various symptoms in a high percentage of cases. Such claims, if true, may tend to minimize the importance of errors of a statistical nature. In this survey we are not directly interested in these considerations. We might suggest, in passing, that the occurrence of eosinophiles in some of these specimens might indicate the presence of an allergic reaction.

These appendectomies cover the ten-year period from January 1, 1928, to January 1, 1938. For purposes of discussion, classifications have been used as indicated by the headings in Table I.

In assembling the figures in this study an attempt has been made to verify the diagnoses as they appear on the charts. The operator may, through carelessness or for other reasons, introduce inconsistencies into his records. If his examination and findings clearly indicate this, the diagnosis is corrected accordingly. Surgeons often disregard the findings of the pathologist. Thus, one may make a clinical diagnosis of acute appendicitis and an operative diagnosis of acute or subacute appendicitis. The pathologist may diagnose normal appendix, while the surgeon signs the case out with a final diagnosis of acute or subacute appendicitis.

The reader will notice certain cases labeled "chronic appendicitis (I.A.)." Where the report of the pathologist is "chronic appendicitis," but his description (gross and microscopical) of the pathologic findings does not reveal definite pathology, the designation I.A. is added to indicate "innocent appendix." Many pathologic diagnoses of "chronic appendicitis" appear on charts as a carry-over from days of ultrascientific classification or as a euphemism. This policy has now been corrected in our laboratory. No appendix is noted as normal or innocent if its wall is thickened or stenotic, nor if any of them contain

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fecaliths. Appendices showing only some injection of the serosa or containing soft fecal material are classed as innocent.

No doubt a dozen different workers going over these same charts would get a dozen different sets of figures; but they would not vary much. Our results, working independently on separate groups, show that the pathologist (A. A. K.) considers three-quarters of the so-called chronic appendicitis specimens as innocent, whereas the other of us (A. S. A.) places only 2 out of 3 in that classification. Other percentages are practically identical.

We can see no advantage here in expressing percentages beyond the decimal and shall, therefore, use whole numbers only.

TABLE I  
LABORATORY DIAGNOSIS

Showing the laboratory (pathologist's) diagnoses as compared to the clinical or preoperative diagnoses. (Thus, of the 142 cases diagnosed clinically as subacute, 17 were found to be subacute, 22 were chronic, etc. Similarly, of the 90 cases found pathologically to be subacute, 17 had preoperatively been diagnosed subacute, 18 had been diagnosed as chronic, etc.)

CLINICAL DIAGNOSIS	ACUTE, NO RUPTURE	ACUTE, RUPTURE	SUB-ACUTE	CHRONIC	CHRONIC OBLITERATIVE	CHRONIC (I.A.)*	NORMAL	TOTAL (CLINICAL DIAGNOSIS)
Acute, not ruptured	604	37	48	62	27	101	97	975
Acute, ruptured	36	32	1	2	0	4	1	75
Subacute	14	1	17	22	7	59	22	142
Chronic	13	0	18	89	44	210	111	485
Normal (not mentioned)	5	1	6	30	36	80	263	421
Total (laboratory diagnosis)	672	71	90	205	114	453	493	2098

\*Innocent appendix.

#### CLINICAL DIAGNOSIS

*Acute (Including Ruptured) Appendicitis.*—Of the 2,098 appendectomies, 1,050, or one-half, were diagnosed as acute. Of these, 709, or 67 per cent, were actually acute. This means that 2 appendices out of 3 will confirm a clinical diagnosis of acute appendicitis.

Conversely, we note that there were actually 743 cases of acute appendicitis in the entire series. Of these, 709, or 95 per cent, were diagnosed preoperatively as acute. This means that if one has acute appendicitis, the chances are 95 out of 100 that the physician will ultimately recognize it as such.

*Significant (Acute and Subacute) Appendicitis.*—There seems little use in considering the diagnosis of subacute appendicitis in the same way. We may, however, group acute and subacute appendicitis together, as including those cases in which there is definite and significant disease. Doing this, we find that of 1,192 clinical diagnoses of acute and subacute appendicitis, 790, or 66 per cent, were actually acute or subacute. Thus, if we diagnose significant pathological change in the appendix, the chances are only 2 out of 3 that we will be right.

Contrariwise, we see that there were in the series 833 cases of acute and subacute appendicitis, of which 790, or 95 per cent, were diagnosed clinically as such. Here again, with significant pathologic change in the appendix, the chances are 95 out of 100 that the physician will correctly determine it. This indicates that our chief problem, statistically, lies not in recognizing acute or subacute appendicitis, but in recognizing those things with which they may be confused.

*Chronic Appendicitis.*—The field of chronic appendicitis involves much difference of opinion. Some claim that the entity does not exist. Some claim that it does exist, but should not include the obliterative types. So far as we are concerned, for these purposes, we shall group all cases of chronic appendicitis together, including with the normal cases those diagnosed as chronic but considered by us as having no pathology that could reasonably be supposed to give clinical symptoms, that is, those specimens designated by us as innocent appendices (I.A.).

The practical significance of obliterative appendicitis, however, may be indicated by Table II.

TABLE II  
OBLITERATIVE APPENDICITIS

CLINICAL DIAGNOSIS		PATHOLOGICALLY OBLITERATIVE
Chronic appendicitis	485 cases	44 cases, or 9 per cent
Normal appendicitis	421 cases	36 cases, or 9 per cent

We see from Table II that there is the same percentage of obliterative appendices among "normal" individuals as among those thought to have chronic appendicitis. Therefore, we may suspect that the obliterative appendix causes no more trouble than the normal appendix.

We note in passing that obliterative appendicitis is not a well-recognized condition by the surgeon. Although it occurred 141 times, he mentioned it only 10 times.

Of the 485 diagnoses of chronic appendicitis, then, there were 133, or 27 per cent, that were actually sufficiently abnormal to justify the belief that they may have given rise to symptoms. Thus, the chances are about 1 out of 4 that a diagnosis of chronic appendicitis will be verified by the pathologist. It is true that occasionally a diagnosis of chronic appendicitis will lead to disclosure of an acute or subacute appendicitis. This elevates the level of accuracy of diagnosis to 34 per cent. This means that when we diagnose chronic appendicitis, the chances are only 1 in 3 that the appendix will be in any way abnormal.

Turning this around again, we note that of 319 cases of chronic appendicitis, 133, or 42 per cent, were diagnosed clinically as such. Thus the chances of our accurately recognizing chronic appendicitis clinically are 5 out of 12. Hence we are only slightly more likely to identify it when present than to diagnose it incorrectly when it is not present; and we are more likely to miss it than to recognize it when it is present.

In the "normal" group of 421 cases we find that the incidence of chronic appendicitis is about 16 per cent. We may thus conceive that of every 6 per-



sons on the street, 1 has chronic appendicitis from the standpoint of the pathologist. But the incidence of chronic appendicitis in those diagnosed as having it is only 27 per cent, or about 1 in 4. Our error would thus be fairly small if we would choose our cases of chronic appendicitis at random, without attempt at diagnosis.

We may put this another way. If the conditions found at operation upon the chronic appendicitis patient are the same as those found at operation upon many "normal" patients, then the so-called symptoms of chronic appendicitis may not arise from appendicitis, or even from the appendix, at all. Clinically, there may be no chronic appendicitis, or only a rare one. This fact will explain some of the conclusions to follow.

Interesting for comparison is Shelley's series<sup>1</sup> where, among 1,890 appendices removed from patients without sign or symptom of appendiceal disease, two-thirds showed definite pathologic change.

*Ruptured Appendicitis.*—We feel that there may be little point in calculating the accuracy of diagnosis in cases of rupture. We suspect that neither surgeon nor pathologist goes to great pains to distinguish acute nonruptured from acute ruptured appendicitis, at times merely noting the acute form when rupture obviously existed. However this may be, our records show that of 75 clinical diagnoses of rupture, 61, or 81 per cent, were actually ruptured according to operative diagnosis and 32, or 43 per cent, according to laboratory diagnosis.

Conversely, of 118 cases diagnosed as ruptured operatively, 61, or 51 per cent, were so diagnosed clinically, while the laboratory confirmed the diagnosis in 64 cases, or 54 per cent of all cases.

*Appendicitis of All Types (as a Group).*—In this series of 2,098 appendectomies, 1,677 appendices, or 80 per cent, were diagnosed preoperatively as abnormal. This means that 1 appendectomy in 5 is an "incidental" appendectomy. Of the 1,677 diagnosed clinically as abnormal, 1,152, or 69 per cent, were recognized pathologically as abnormal. In other words, if we think that an appendix is abnormal in any way, the chances are about 2 in 3 that we are right. Of the 1,677 cases diagnosed as appendicitis clinically:

- 737, or 44 per cent were acute by laboratory examination.
- 84, or 5 per cent were subacute by laboratory examination.
- 253, or 15 per cent were chronic by laboratory examination.
- 603, or 36 per cent were normal by laboratory examination.

We may compare this to Aschoff's series<sup>2</sup> of 847 cases diagnosed as appendicitis with no evidence of it pathologically in 35 per cent. Sappington and Horneff<sup>2</sup> present an even worse picture with a series of 937 cases diagnosed as appendicitis and 565, or 60 per cent, pathologically normal. Other statistics present errors of 2 per cent, or even nothing at all.<sup>3</sup>

We may deduce from Table I that the chance of diagnosing "diseased appendix" incorrectly (36 per cent) is almost twice the chance of diagnosing "normal appendix" incorrectly (19 per cent). Thus it is twice as easy clinically for us to misdiagnose a normal appendix as a diseased one.

## OPERATIVE DIAGNOSIS

*Significant (Acute and Subacute) Appendicitis.*—Referring to Table III, of 892 diagnoses of acute appendicitis made at the operating table, 711, or 80 per cent, were actually acute according to the pathologist. If we combine subacute with acute cases as previously, there were 1,090 operating room diagnoses of definite and significant appendicitis. The pathologist agreed in 797, or 73 per cent, of these cases. This means that for every 4 appendices that we diagnose at the operating table as being significantly diseased, we are wrong once. It is also interesting to note that of these 1,090 surgical diagnoses of acute and subacute appendicitis, 83 were chronic and 210 (1 in 5) were actually normal.

TABLE III

## LABORATORY DIAGNOSIS

Showing the laboratory diagnoses as compared to the surgical or operative diagnoses. (Thus, of 198 cases diagnosed as subacute at the operating table, 28 were subacute, 30 were chronic, etc. Similarly, of the 91 cases diagnosed as subacute by the laboratory, 28 were thought to be subacute at the operating table, 20 were thought to be chronic, etc.)

OPERATIVE DIAGNOSIS	ACUTE, NO RUPTURE	ACUTE, RUPTURE	SUB-ACUTE	CHRONIC	CHRONIC OBLITERATIVE	CHRONIC I.A.	NORMAL	TOTAL (OPERATIVE DIAGNOSIS)
Acute, not ruptured	590	7	39	32	9	56	41	774
Acute, ruptured	50	64	1	2	0	0	1	118
Subacute	18	0	28	30	10	64	48	198
Chronic	11	0	20	125	57	285	199	697
Chronic obliterative	0	0	0	1	7	1	1	10
Normal	2	0	3	16	31	46	203	301
Total (Laboratory diagnosis)	671	71	91	206	114	452	493	2098

Conversely, we note that of the 742 cases of pathologically acute appendicitis, 711, or 96 per cent, were diagnosed as such operatively. Combining acute with subacute, of the 833 significantly inflamed organs, 797, or 96 per cent, were recognized. Thus our chances of correctly recognizing definite and significant inflammation of the appendix at the operating table are 96 out of 100.

*Chronic Appendicitis.*—Similarly, we diagnosed chronic appendicitis operatively 707 times when the condition existed 190 times, with an accuracy of 27 per cent, and we recognized chronic appendicitis 190 times out of 320 actual cases, with an accuracy of 59 per cent. Operatively, as clinically, we misdiagnose the normal appendix twice as readily as the diseased one.

*Appendicitis of All Types (as a Group).*—Of the 1,153 actually abnormal appendices, only 52 were diagnosed as normal at the operating table. But of the 945 normal appendices, 696 were diagnosed as abnormal. This means that our diagnosis of the pathologically normal appendix as made at the operating table is worthless and that we are more likely to be wrong than right by 3 out of 4. How much of this error is due to insincerity or carelessness and how much to inability must remain for each individual to decide. The error comes chiefly, of course, in the field of chronic appendicitis.

A reason for this lack of correlation between laboratory and operative findings becomes more clear when we study Table IV.

TABLE IV

## OPERATIVE DIAGNOSIS

Showing the operative or surgical diagnoses as compared to the clinical diagnoses. (Thus, of the 140 cases diagnosed clinically as subacute, 83 were thought to be subacute at the operating table, 39 were thought to be chronic, etc. Similarly of the 198 cases thought to be subacute at the operating table, 83 had been diagnosed subacute clinically, 21 had been diagnosed chronic clinically, etc.)

CLINICAL DIAGNOSIS	ACUTE, NO RUPTURE	ACUTE, RUPTURE	SUB-ACUTE	CHRONIC	CHRONIC OBLITERATIVE	NORMAL	TOTAL (CLINICAL DIAGNOSIS)
Acute, not ruptured	736	51	82	56	1	49	975
Acute, ruptured	9	61	1	2	0	3	76
Subacute	13	2	83	39	0	3	140
Chronic	8	1	21	426	6	17	479
Normal (not mentioned)	5	3	11	184	4	221	428
Total (operative diagnosis)	771	118	198	707	11	293	2,098

Thus, with 1,191 cases diagnosed clinically as acute or subacute, 1,038 were so classified at the operating table, while only 790 were acute or subacute. With 479 diagnosed chronic clinically, 432 were diagnosed chronic operatively and 133 were actually chronic. With 428 presumed to be normal clinically (patient operated for other conditions entirely) 221 were diagnosed normal at operation, while 343 were actually normal.

Let us look at it this way:

Clinically normal 428	Operatively normal 221	Pathologically normal 343
Clinically chronic 479	Operatively normal 17	Pathologically normal 321

Whether the clinical diagnosis is normal or chronic, the chances of the appendix being actually normal are almost the same, while the chances of diagnosing the normal appendix incorrectly at operation are about 12 to 1 in favor of the operator's preoperative diagnosis. We may, therefore, conclude that the operator is governed mainly by his clinical diagnosis in distinguishing normal from chronically altered appendices, and that these two conditions are actually indistinguishable in the great majority of cases. The same holds true with other types of appendicitis, though to a lesser degree.

## CAUSES OF ERROR

One cause of operative error has been discussed, namely, suasion of the operator in favor of his preoperative diagnosis. This factor is further evidenced, first, by the relative tendency to diagnose an appendix as normal rather than chronic when other pathology is present and, second, to make an intermediate diagnosis when the clinical diagnosis is wide of its mark.

Errors in clinical diagnoses of *acute appendicitis* occur mainly in females and are due to ruptured ovarian follicles and cysts, pelvic inflammatory disease,

ectopic pregnancy, and other gynecologic conditions. Of 61 cases operated upon for acute appendicitis, where a normal appendix was found at operation, 26 come in this group. Six were due to gall bladder disease, 7 to perityphlitis,\* 2 to ruptured duodenal ulcer, 2 to intestinal obstruction, 2 to Meckel's diverticulitis, 1 each to lymphoma, gastro-enteritis, mesenteric adenitis, and coronary disease, and 12 to undetermined cause.†

The greatest cause of error in general appears to be nonsurgical conditions. Where *chronic* appendicitis is diagnosed alone clinically, and appendicitis is not discovered at operation, the great majority of cases will reveal no lesion to exploration as performed at this hospital. It may be noted, moreover, that the usual incision for chronic appendicitis, as done by the hospital staff, is not a muscle-splitting incision, but some form of rectus or midline incision.

A potent factor in misleading surgeons in diagnosis is undoubtedly their failure to possess accurate criteria of appendicitis. Reference is frequently made to veils, kinks, serosal injection, retrocecal position, etc., as evidencing appendicitis and justifying the diagnosis thereof.

#### MORTALITY

In collecting these statistics we have noted fatal cases, though this may not be directly in line with our survey. There were in all 26 deaths, distributed as follows:

Acute ruptured appendicitis	16
Acute nonruptured appendicitis	10
Appendix normal or innocent	9

Of the 9 persons who died from complications other than appendicitis, 2 were operated upon due to erroneous diagnosis of acute appendicitis. One was operated upon for chronic appendicitis and hemorrhoids, and an innocent appendix was found. The other 6 were "incidental" appendectomies, the patients dying of the primary lesion or a complication of it.

It will be noted that all but 1 of the deaths in operations for appendicitis, where no other serious condition existed, were due to *acute* appendicitis only. Mortality for operation itself has, therefore, been less than 1 in 2,000. Mortality for acute appendicitis with rupture was about 1 in 7, using the operative diagnosis of rupture as a basis, whereas the mortality in acute nonruptured cases was about 1 in 67.‡ Our principal concern for improving the death rate is, therefore, with these 2 groups, and our principal concern in avoiding unnecessary operations has to do with morbidity and economy.

The total mortality in acute appendicitis has been reduced 65 per cent (from 8 per cent to 3 per cent) in the second five-year period as compared to the first five-year period (Table V), while appendectomies have increased twice as rapidly as the general population (50 per cent) and at the same rate as increase in staff membership§ (110 per cent) over the ten-year period. It may

\*This term is used to indicate some type of inflammation in the region of the cecum and of unknown origin.

†Included here are some cases called appendicitis by the surgeon and one called spastic colitis.

‡Bower<sup>3</sup> states: "When the serous membrane is intact, 1 in 183 dies; when it is ruptured, 1 in 8 dies."

§Total staff membership 50 on January 1, 1928, and 105 on January 1, 1938.

well be that the doubling of operations for acute appendicitis accounts for the reduction in ruptures and mortality.

TABLE V  
MORTALITY BY YEARS

YEAR	CASES OF PATHOLOGICALLY ACUTE APPENDICITIS (ALL TYPES)	TOTAL NUMBER OF DEATHS	NO. OF DEATHS WITH RUPTURE	PERCENTAGE OF DEATHS IN ACUTE CASES (ALL TYPES)
1928	43	1	0	2
1929	68	6	3	9
1930	69	5	2	7
1931	49	4	1	8
1932	67	7	6	10
1933	65	1	0	2
1934	60	0	0	0
1935	83	3	2	4
1936	120	5	1	4
1937	119	3	2	3

#### PROGRESS IN DIAGNOSIS

Mortality rates may be explained in part upon the basis of diagnosis. Thus, if diagnosis becomes more accurate, it may appear to decrease the mortality by adding cases of appendicitis formerly misdiagnosed, or it may increase the mortality by differentiating cases previously included through error. Actually, the clinical diagnosis of acute appendicitis for each of the ten years at this hospital has remained practically unchanged, as shown in Table VI.

TABLE VI  
DIAGNOSIS OF ACUTE APPENDICITIS BY YEARS

Showing the accuracy of one type of diagnosis as compared with another for each of the ten years covered.

YEAR	DIAGNOSED CLINICALLY	CONFIRMED OPERATIVELY	% ACCURACY	DIAGNOSED CLINICALLY	CONFIRMED BY LABORATORY	% ACCURACY	DIAGNOSED BY LABORATORY	PREDICTED CLINICALLY	% ACCURACY	DIAGNOSED OPERATIVELY	CONFIRMED BY LABORATORY	% ACCURACY
1928	59	46	78	59	40	68	43	40	93	49	40	82
1929	105	84	80	105	66	63	68	66	97	87	65	75
1930	89	73	82	89	65	73	69	65	94	79	67	85
1931	68	58	85	70	48	69	49	48	98	59	49	83
1932	97	76	78	97	66	68	67	66	99	78	65	83
1933	93	73	79	93	64	69	65	64	99	76	64	84
1934	92	72	78	92	60	65	60	60	100	74	59	79
1935	113	96	86	113	78	69	83	78	94	100	80	80
1936	163	140	86	162	110	68	120	110	92	149	110	74
1937	170	139	82	170	112	66	119	112	94	141	112	80
Total	1,049	857	82	1,050	709	68	743	709	95	892	711	80

We do not know how many cases of appendicitis and nonappendicitis of various types were seen by the staff but neither operated upon nor even diagnosed. For this reason, we cannot determine all clinical error. Bedside research is badly needed for such information. We may infer a considerable delay somewhere from the fact that of 742 acute cases, 114, or 15 per cent (1 in 7), were ruptured at operation. However, 95 per cent of acute cases were ultimately

diagnosed as acute. The bulk of the remaining acute cases were diagnosed as appendicitis of some type, and operation was nearly always advised. Error in failure to *ultimately* recognize appendicitis must, therefore, be small. The tragedies come in delay. This delay may stem from our inability to recognize low-grade appendicitis accurately.

We note, however, the many diagnoses of acute appendicitis where acute appendicitis did not exist. The laboratory confirms our clinical diagnosis in only 2 cases out of 3. Until we can observe the appendix in the opened abdomen and correctly state whether or not it is significantly inflamed with an accuracy greater than 3 in 4, we may be unable to improve our recognition of the acutely inflamed appendix in the closed abdomen. Until our preoperative diagnosis does improve there must be unnecessary operations on the one hand, and on the other, unnecessary delay, ruptures, and deaths. Statistically, our diagnostic acuity has remained unchanged year after year (Table VI).

#### MULTIPLE DIAGNOSES

Special interest and importance attaches to those operations in which multiple clinical diagnoses are made. One wants to know whether the laws of chance justify a laparotomy, for chronic appendicitis especially, and whether fortuitous findings are likely to enhance the value of such surgery.

TABLE VII

#### MULTIPLE LESIONS

Showing the actual findings in operations for multiple lesions as compared to the clinical diagnoses. (Thus, of 226 cases diagnosed as having chronic appendicitis with other abdominal lesions, 8 had chronic appendicitis alone, 30 had no lesion at all, etc.)

CLINICAL DIAGNOSIS	ACTUAL FINDINGS (OPERATIVE AND LABORATORY)					TOTAL (CLINICAL DIAGNOSIS)
	CHRONIC APPENDI- CITIS WITHOUT OTHER LESIONS	CHRONIC APPENDI- CITIS WITH OTHER LESIONS	NORMAL APPENDIX WITHOUT OTHER LESIONS	NORMAL APPENDIX WITH OTHER LESIONS	ACUTE OR SUBACUTE APPENDI- CITIS WITH OR WITH- OUT OTHER LESIONS	
Chronic appendicitis without other lesions	69	8	138	18	22	255
Chronic appendicitis with other lesions	8	56	30	128	4	226
Normal appendix (opera- tion for other lesions)	5	62	21	322	13	423
Acute or subacute ap- pendicitis (with without other lesions)	97	22		77	792	1,194

them. In some instances, as with small corpus luteum or other ovarian cysts, leading merely to puncture, the condition is disregarded.

We note that there were 255 cases with the clinical diagnosis of chronic appendicitis unaccompanied by any other diagnosis justifying laparotomy. Of this group:

- (a) Diagnosis was exactly verified in 69 cases, or 27 per cent (about 1 in 4).
- (b) Diagnosis of chronic appendicitis was verified but other lesions were found in 8 cases, or 3 per cent (about 1 in 32).
- (c) Diagnosis was entirely in error but other lesions were found in 40 cases, or 16 per cent (about 1 in 6)
- (d) There was no lesion whatever to justify the laparotomy in 138 cases, or 54 per cent (about 1 in 2).

Coming to the cases diagnosed as having chronic appendicitis accompanied by other intra-abdominal lesions (including hernias), we see that there were 226. Of these:

- (a) Diagnosis was exactly verified in 56 cases, or 25 per cent (1 in 4).
- (b) Chronic appendicitis alone was found in 8 cases, or 4 per cent (1 in 28).
- (c) Diagnosis was in error but other lesions were found in 132 cases, or 59 per cent (about 6 in 10).
- (d) There was no lesion whatever to justify the laparotomy in 30 cases, or 13 per cent (about 1 in 8).

It appears that we can recognize chronic appendicitis alone and chronic appendicitis accompanied by other intra-abdominal lesions with equal facility (1 in 4), and that the "other lesions" considered in each case are as likely to be missing when diagnosed as present as when not diagnosed (1 in 30).

When no chronic appendicitis exists, moreover, we are likely to find some other lesion in only 22 per cent, or 1 out of 5 cases, if we didn't expect one previous to operation. If we did expect another lesion previous to operation and failed to find the chronic appendicitis itself, our chances are 82 per cent, or 5 in 6 of finding another lesion.

Noteworthy is the fact that of those operations for chronic appendicitis alone, over half may be described as useless, whereas, if we think that other lesions accompany the chronically changed appendix, 1 operation in 8 may be designated as useless.

Noteworthy also is the fact that there are also almost as many diagnoses of chronic appendicitis with as without other abdominal lesion. This suggests that extra-appendiceal lesions are not active in the production of appendicitis.

Turning to those operations where we believe or assume that the appendix is normal, we note that there are 423. It is actually normal in 343, or 81 per cent, of the cases (4 in 5). In 21 cases, or 5 per cent (1 in 20), no pathologic condition was found, i.e., the operation may be described as useless. As this error is less than that for the diagnosis of appendicitis as a whole, 36 per cent, it means that appendicitis is 7 times as easy to misdiagnose as other abdominal lesions (as a group).

One final observation: Of the 1,194 cases thought clinically to have acute or subacute appendicitis (with or without other lesions), but not having it, 99, or 8 per cent, had lesions elsewhere, while 97, or 8 per cent, had chronic appendicitis alone, and 206, or 17 per cent, had no intra-abdominal lesion whatever to justify laparotomy, so far as was found.

#### SUMMARY

Following are some of the conclusions resulting from this statistical study.

1. Only 2 appendices out of 3 will confirm a clinical diagnosis of acute appendicitis.

2. Of appendices actually acute, 95 per cent will ultimately be correctly diagnosed clinically.

3. The foregoing statements hold true when we group acute and subacute appendicitis together.

4. Chronic obliterative appendicitis causes no more trouble clinically than the normal appendix.

5. The chances are 1 out of 4 that a diagnosis of chronic appendicitis will be verified by the pathologist.

6. A diagnosis of chronic appendicitis means that the chances are 1 in 3 that the appendix is in any way abnormal.

7. When chronic appendicitis actually exists, the chances are about 5 in 12 that we will recognize it clinically; i.e., the chances are that we will not recognize it when we meet it.

8. One "normal" person in 6 has a pathologically "chronic appendicitis."

9. Thirty-five per cent (one-third) of all individuals operated upon for diseased appendix have a normal appendix.

10. It is twice as easy for us to misdiagnose a normal appendix clinically as a diseased one.

11. For every 4 appendices that we diagnose as significantly diseased (acute or subacute appendicitis) at the operating table, we are wrong once, and more than 1 such appendix in 5 will actually be normal.

12. Of appendices actually acutely or subacutely inflamed, 96 per cent will be recognized as such at the operating table.

13. When we think that an appendix is chronically inflamed at operation, we are correct in about 1 case in 4.

14. When we see a chronically inflamed appendix at the operating table, we can recognize it about 6 times out of 10.

15. Operatively, as clinically, it is twice as easy for us to misdiagnose the normal appendix as it is the diseased appendix, and when the appendix is normal our operative diagnosis will be wrong 3 times out of 4.

16. Whether the clinical diagnosis is "normal" or "chronic," the chances of the appendix being normal are almost the same, while the chances of diagnosing the normal appendix incorrectly at operation are about 12 to 1 in favor of the operator's preoperative diagnosis.

17. The chief cause of error in the clinical diagnosis of acute appendicitis is some gynecologic condition. Next in importance come "no surgical lesion," gall bladder disease, and inflammation in the region of the cecum, in that order.



18. Where chronic appendicitis is diagnosed alone clinically and not found, the great majority of cases will reveal no lesion to exploration.

19. The mortality in acute ruptured appendicitis over a ten-year period was 1 in 7 and that of the nonruptured acute cases was 1 in 67.

20. In ten years the diagnostic acuity of the staff of the Santa Monica Hospital has not changed, as regards appendicitis. Mortality rate has improved, along with increased frequency of operation.

21. Error in failure to ultimately recognize acute appendicitis is small (5 per cent). Delay in diagnosis and operation are the important factors. Because of it, 1 acutely inflamed appendix in 7 was ruptured at operation.

22. These statistics must be supplemented by data obtained before the patient's entry into the hospital to determine causes of delay, clinical error, etc.

23. We recognize chronic appendicitis alone and chronic appendicitis accompanied by other intra-abdominal lesions with equal facility, and accompanying lesions are as easily ruled out as recognized.

24. When we diagnose chronic appendicitis and it is absent at the operating table, we are likely to find some other lesion in only 1 case in 4 if we did not expect it before operation, and in 5 cases out of 6 if we did expect it.

25. Of those operations for chronic appendicitis alone, over half reveal no pathologic condition whatever. In operations for chronic appendicitis with other intra-abdominal lesions, 1 case in 10 reveals no pathologic condition.

26. Extra-appendiceal lesions are not active in producing appendicitis.

27. When the appendix appears to be normal clinically (operation for other cause entirely), it is actually normal in 4 cases out of 5. In these cases, no pathologic condition at all was found in only 3 per cent.

28. Appendicitis is 7 times as easy to misdiagnose as other abdominal conditions (as a group).

29. Of cases thought clinically to have acute or subacute appendicitis, 1 case in 6 revealed no pathologic condition of any kind at operation.

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## THE STABILITY OF ASCORBIC ACID IN BLOOD\*

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WE HAVE been engaged in an extensive study of vitamin C metabolism in health and in disease, with particular reference to rheumatic fever and rheumatoid arthritis.<sup>1, 2</sup> In previous work we have shown that the plasma level of reduced ascorbic acid during fasting afforded an accurate index of the immediate nutritive status relative to vitamin C.<sup>3</sup> In this work we have employed the method of Farmer and Abt,<sup>4, 5</sup> and have investigated various factors which might influence the stability of vitamin C in the blood. This seemed particularly pertinent in view of the criticism of the method made by Pijoan and co-workers<sup>6, 7</sup> who reported that the ascorbic acid of the plasma undergoes rapid destruction after the blood has been withdrawn from the body. This was not in accord with our own experience. Pijoan also claimed that this loss could be prevented by the addition of potassium cyanide to the blood. Following this report we undertook a systematic study of the stability of ascorbic acid in blood and of the influence of cyanide upon this. While this work was in progress Farmer and Abt,<sup>8</sup> Friedman, Rubin, and Kees,<sup>9</sup> and Cushman and Butler<sup>10</sup> published reports which failed to show a stabilizing influence with cyanide. Furthermore, Farmer and Abt showed that potassium cyanide per se was capable of decolorizing 2,6 dichlorophenolindophenol and that it produced an increase in the apparent ascorbic acid values when added to blood. In the present paper further evidence bearing on the effect of potassium cyanide on 2,6 dichlorophenolindophenol and upon the stability of ascorbic acid in blood and plasma is reported. In addition, some experiments on the comparative stability of ascorbic acid in whole blood and plasma are presented.

Typical experiments showing the comparative stability of ascorbic acid in whole blood and plasma are shown in Table I. In these experiments 40 to 50 c.c. of blood were drawn from human subjects by venepuncture and distributed in 4 test tubes containing dry powdered sodium oxalate. Two of the portions of blood were centrifuged immediately, the plasma separated, and an aliquot portion of the latter was subjected to analysis as soon as possible. One test tube each of whole blood and plasma were then set aside at room and refrigerator temperatures, respectively. At the end of the time intervals indicated, analyses were carried out on the plasma of each of the 4 specimens. Deproteinization was carried out on 1 c.c. portions of each plasma according to the method of Pijoan and Eddy<sup>11</sup> and 2 c.c. portions of the filtrate were used for titration. The results recorded in Table I show that different bloods exhibit differences in their

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loss of reducing capacity on standing. In some cases the presence of red blood cells appears to exert a protective action on the ascorbic acid of the plasma, while in others this effect is absent. These results are somewhat at variance with those reported by Kellie and Zilva,<sup>12</sup> and Borsook and co-workers,<sup>13</sup> who reported that red blood cells exerted a protective influence on ascorbic acid. From one observation on dog blood, Barron, Barron, and Klemperer<sup>14</sup> concluded that erythrocytes do not exert any influence on the rate of oxidation of the ascorbic acid of the plasma.

TABLE I

THE STABILITY OF ASCORBIC ACID IN PLASMA AND WHOLE BLOOD AT ROOM AND REFRIGERATOR TEMPERATURE

TEMPERATURE		REFRIGERATOR		ROOM	
BLOOD SPECIMEN	TIME OF STANDING	PLASMA	WHOLE BLOOD	PLASMA	WHOLE BLOOD
B.	Hours	Mg. per cent	Mg. per cent	Mg. per cent	Mg. per cent
	Immediate	1.60	—	—	—
	0.6	1.41	1.60	1.38	1.60
	1.6	1.41	1.60	1.35	1.57
	3.0	1.41	1.60	1.23	1.54
	20.0	0.99	1.38	0.62	1.35
G.	Immediate	0.96	—	—	—
	1	0.96	0.96	—	0.96
	2	0.96	0.96	—	0.93
	3	0.93	0.96	—	0.90
	24	0.72	0.78	—	0.54
L.	Immediate	1.39	—	—	—
	0.5	1.30	—	1.30	—
	1.5	1.30	1.33	1.28	1.30
	3.0	1.30	1.30	1.28	1.28
	6.0	1.25	1.30	1.19	1.25
R.	Immediate	1.40	—	—	—
	1	1.37	1.37	—	—
	2	1.28	1.22	—	—
	3	1.16	1.22	—	—
	5	1.16	1.22	—	—
	24	0.91	1.22	—	—

However, the main point brought out by the data in Table I is the fact that whole blood kept at refrigerator temperature suffers but little loss in its ascorbic acid content over a period of two to three hours. We have examined some bloods which showed little or no loss under these conditions for periods as long as twenty-four hours. Cushman and Butler<sup>10</sup> have made similar observations.

After the appearance of Pijoan and Klemperer's paper we attempted to confirm their work. Since we had no potassium cyanide at hand, we employed sodium cyanide. Our observations showed no increase in the stability of the ascorbic acid of the plasma in the presence of sodium cyanide. Unlike potassium cyanide, sodium cyanide itself produced no decolorization of the dye 2,6 dichlorophenolindophenol. Three experiments were performed in which 40 to 50 c.c. of blood were drawn from three different individuals and 5 c.c. portions were distributed in tubes containing (1) sodium oxalate and (2) 15 to 20 mg. of a mixture of sodium oxalate and sodium cyanide in the proportion of two parts of the former and one of the latter. One sample of each series was centrifuged, and analyses were carried out on 1.0 c.c. quantities of the plasma according to the method described earlier. The remaining tubes of blood were kept at refrigerator

temperature (9° to 10° C.) and subsequently analyzed after varying intervals of time. In each case the time is reckoned from the moment the deproteinizing agent (metaphosphoric acid) was introduced into the plasma. The results of the experiments are shown in Table II. These are in accord with the observations of Farmer and Abt, Friedman, Rubin, and Kees, and Cushman and Butler. They show that the addition of sodium cyanide to blood offers no protection against the oxidation of the vitamin.

TABLE II  
INFLUENCE OF NACN ON THE ASCORBIC ACID VALUES OF PLASMA  
(Whole blood left at refrigerator temperature)

BLOOD SPECIMEN	TIME OF STANDING	WITHOUT NACN	WITH NACN
R	Hours	Mg. per cent	Mg. per cent
	0	1.02	0.96
	2.5	0.96	0.96
	5.0	0.90	0.85
	24.0	0.79	0.79
G.	0	1.27	1.30
	2.5	1.21	1.24
	5.0	1.15	1.09
P	0	0.67	0.67
	2.7	0.60	0.60
	4.0	0.60	0.60
	27.5	0.43	0.49

Comparable studies were made with potassium cyanide. Ten milligram quantities of several lots of C.P. potassium cyanide dissolved in 2 c.c. of 2.5 per cent metaphosphoric acid were found to decolorize about 1.5 to 1.6 c.c. of dye with an ascorbic acid equivalency of 0.012 mg. per c.c. In addition, two other lots of sodium cyanide were tested in quantities 5 to 6 times as large as those reported for potassium cyanide, and the titration values were found to be of the same order of magnitude as blank determinations on equivalent volumes of metaphosphoric acid alone. When these larger amounts of sodium cyanide were used, much larger quantities of metaphosphoric acid were necessary because of the alkalinity produced by the former.

These tests led us to suspect that the samples of potassium cyanide might be contaminated with ferrocyanides. Ferrocyanide is listed as a common contaminant of both C. P. sodium and potassium cyanides. Moreover, ferrocyanides may be used in the preparation of cyanides. Tests on small amounts of potassium ferrocyanide dissolved in metaphosphoric acid showed this substance decolorized the "indicator reagent"; 0.86 mg. quantities of potassium ferrocyanide dissolved in 2 c.c. of 2.5 per cent metaphosphoric acid were found to decolorize 0.58 c.c. of dye (1 c.c. of dye = 0.222 mg. of ascorbic acid). Murray<sup>15</sup> lists 0.005 per cent as the maximum limit for ferrocyanides as an impurity of C.P. cyanides of sodium and potassium. Another common contaminant of cyanides is thiocyanate. This substance was found not to decolorize the dye used for the titration of vitamin C.

Further evidence that the decolorization of 2,6 dichlorophenolindophenol by potassium cyanide is attributable to the presence of ferrocyanides as an impurity was obtained by carrying out the Prussian blue test on both the sodium

and potassium cyanides which we had available. Five-tenths to 1 Gm. quantities of the different samples of potassium cyanide were found to give strong positive Prussian blue reactions, while similar quantities of the various sodium cyanides failed to give this test.

During the routine analysis of a large number of blood specimens we had observed that when an appreciable amount of hemolysis was present the ascorbic acid values were usually low. In this connection it has been reported by Gabbe,<sup>16</sup> Kellie and Zilva,<sup>12</sup> van Eekelen,<sup>17</sup> Emmerie and van Eekelen,<sup>18</sup> Berend and Fischer,<sup>19</sup> and Klodt,<sup>20</sup> that in the presence of oxyhemoglobin a part of the ascorbic acid is lost or destroyed. Fischer<sup>21</sup> has observed that the rate of disappearance of ascorbic acid in the presence of hemoglobin is extremely rapid. According to the work of Gabbe,<sup>16</sup> van Eekelen,<sup>17</sup> and Borsook and co-workers,<sup>13</sup> if whole blood or red blood cell suspensions are deproteinized with acid precipitants, little or none of the ascorbic acid which is present is found in the resulting filtrate. The release of hemoglobin from the red blood cells during the process of deproteinization appears to account for this change.

TABLE III

INFLUENCE OF HEMOLYSIS AND NACN ON ASCORBIC ACID VALUES OF PLASMA

BLOOD SPECIMEN	TIME OF STANDING	PLASMA WITHOUT HEMOLYSIS	PLASMA + 0.1 C.C. HEMOLYZED BLOOD PER C.C.	
			WITHOUT NACN	WITH NACN
	Minutes	Mg. per cent	Mg. per cent	Mg. per cent
1	0	0.96	-	-
	5	-	0.73	0.73
	20	-	0.68	0.62
	40	0.90	0.62	0.57
	90	0.90	0.62	0.57
	120	0.85	-	-
2	0	1.10	0.75	0.75
	8	1.07	0.75	0.75
	20	-	0.70	0.70
	40	-	0.70	0.70
	60	0.93	0.64	0.64
	90	-	0.64	0.64
	120	0.87	0.58	0.58
	180	0.87	-	-

Relative to this, some studies were carried out to determine the effect of hemolyzed blood upon the ascorbic acid content of plasma. In conjunction with this, a study was made to ascertain whether cyanide prevented the loss or destruction of ascorbic acid which is brought about by hemolysis. The results of several such experiments are listed in Tables III and IV.

In carrying out these experiments blood was drawn from different individuals and collected in oxalated tubes. The plasma was separated and distributed in 3 or 4 tubes. Portions of the original blood or red corpuscles therefrom were hemolyzed with distilled water (one volume of blood or red blood cells to four volumes of water) and added to the plasma in the quantities indicated in the tables. Before the addition of the laked red blood cells sufficient distilled water was added to the control tubes and to the tubes which were to receive the lesser amounts of hemoglobin solution to adjust all preparations to equivalent dilution.

Analyses were carried out immediately after introduction of the hemolyzed blood cells and also after varying time intervals. One cubic centimeter aliquots of the mixtures were employed and the method used was the same as indicated earlier. In the instances where cyanide was tested a sufficient amount of this compound was added to give a concentration of approximately M/50. From an inspection of the data in Tables III to V it can be observed that hemolysis produces an instantaneous disappearance of ascorbic acid which is not prevented by the presence of sodium cyanide. This is followed by a slow decline in the concentration of the vitamin which also occurs in the specimens without hemolysis. The initial loss in ascorbic acid is also observed to increase with increase in the concentration of hemoglobin (Table IV). It is desirable here to point out that hemolysis must be avoided if reliable results are to be obtained for the reduced ascorbic acid values of plasma.

TABLE IV  
INFLUENCE OF INCREASE IN HEMOLYSIS ON ASCORBIC ACID LOSS IN PLASMA

BLOOD SPECIMEN	TIME OF STANDING	PLASMA	PLASMA + 0.1 C.C. HEMOLYZED RED BLOOD CELLS	PLASMA + 0.2 C.C. HEMOLYZED RED BLOOD CELLS	PLASMA + 0.3 C.C. HEMOLYZED RED BLOOD CELLS
	Minutes	Mg. per cent	Mg. per cent	Mg. per cent	Mg. per cent
B'	Immediate	1.27	1.06	0.94	0.80
	15	1.12	0.94	0.88	0.77
	30	1.12	0.97	0.85	0.68
	60	1.06	0.94	0.82	0.68
	120	1.00	0.94	0.82	0.71
G	Immediate	1.10	0.92	0.86	0.74
	15	—	0.92	0.86	0.74
	30	0.98	0.86	0.74	0.68
	60	—	0.86	0.74	0.68
	120	0.92	0.83	0.74	0.65

Further evidence that the "alleged" stabilization of ascorbic acid in blood by potassium cyanide is a mere artifact was obtained by carrying out parallel analyses on blood which had been treated with both sodium and potassium cyanide. The addition of cyanide in quantities equal to or greater than those recommended by Pijoan and Klemperer has been observed to produce hemolysis; this observation has also been reported by Farmer and Abt.<sup>3</sup> The degree of hemolysis has also been found to vary with the concentration of cyanide. Since hemolysis tends to decrease the indophenol titer of the blood, one would expect cyanide (above a certain limiting concentration) to produce such an effect. Actually that is what happens when sodium cyanide is used. However, when potassium cyanide is used, the reducing substance (probably ferrocyanide) which is present in the former as a contaminant tends to compensate in part for the ascorbic acid which is lost as a result of hemolysis. Table V summarizes the data obtained in two experiments in which both sodium and potassium cyanides were used.

In these experiments 50 c.c. quantities of blood were drawn from each of two subjects and placed in centrifuge tubes containing dry powdered sodium oxalate. The blood was centrifuged and the plasma was separated. The procedure at this point was somewhat different in each of the two experiments. In

Experiment 1, 10 c.c. portions of the plasma were placed in each of two test tubes. One cubic centimeter of distilled water was added to one 10 c.c. aliquot of the plasma and 1.0 c.c. of hemolyzed red blood cells to the other. The hemolyzed red blood cells were prepared by washing a portion of the centrifuged corpuscles twice with physiologic saline and adding 4 volumes of distilled water to one volume of corpuscles. Two cubic centimeter aliquot portions of each of these preparations were immediately transferred to centrifuge tubes containing the quantities of each cyanide listed in Table V. Analyses were carried out on these by a method which was essentially the same as that of Farmer and Abt,<sup>5</sup> differing only in the use of 10 per cent metaphosphoric acid instead of 5 per

TABLE V

INCREASE IN APPARENT ASCORBIC ACID BY KCN AND ABSENCE OF INHIBITION OF CYANIDE ON ASCORBIC DESTRUCTION DUE TO HEMOGLOBIN

EXPERIMENT NUMBER	CYANIDE ADDED	NO HEMOLYSIS Ascorbic acid mg. %	HEMOLYSIS Ascorbic acid mg. %
1	None	0.94	0.61
	2 mg. NaCN	0.89	0.61
	4 mg. NaCN	0.80	0.58
	2 mg. KCN	1.09	0.83
	4 mg. KCN	1.23	1.06
2	None	1.28	0.67
	2 mg. NaCN	1.20	0.70
	4 mg. NaCN	1.17	0.70
	2 mg. KCN	1.45	1.14
	4 mg. KCN	1.56	1.23

cent metaphosphoric acid as a protein precipitant. The procedure used in the second experiment is as follows: 2 c.c. portions of the plasma were transferred to 10 centrifuge tubes containing the same quantities of sodium and potassium cyanide as was used in the first experiment. Two-tenths cubic centimeter quantities of hemolyzed corpuscles, prepared as described above, were added to each of 5 of the tubes, followed by 3.8 c.c. of distilled water and 4 c.c. of 10 per cent metaphosphoric acid. In setting up the remaining tubes for analysis the quantities of reagents were the same as recommended by Farmer and Abt. The analyses were carried out as soon as possible after the withdrawal of the blood. It is advisable to emphasize the fact that in the first experiment the hemolyzed corpuscles were added before the addition of the cyanide and in the second after the addition of the cyanide. The results show that the same effect is obtained regardless of whether cyanide is added to the plasma before or after the addition of the hemolyzed blood cells.

Although various explanations have been offered for the mechanism of the action of hemoglobin on the vitamin, it would not be desirable to do more than point these out, since we have no evidence to offer in this connection. Van Eekelen<sup>17</sup> and Kellie and Zilva<sup>12</sup> attribute the loss of ascorbic acid to oxidation of the latter by the oxygen of oxyhemoglobin. Klodt<sup>20</sup> is in accord with this, and claims to have shown that the loss of ascorbic acid parallels the oxygen saturation of the hemoglobin. On the other hand, Fischer, and also Gabbe, are not in agreement with this view. They believe that ascorbic acid is "bound adsorptively" by hemoglobin and does not disappear as a result of oxidation.

## CONCLUSIONS

Whole blood left at refrigerator temperatures does not lose an appreciable amount of its ascorbic acid in two to three hours, and can be expected to give reliable analytical values within this period after withdrawal. In contact with corpuscles the average loss is only 12.6 per cent in twenty-four hours. There is no evidence to indicate that cyanide added to blood protects the vitamin from oxidation. Potassium cyanide itself decolorizes 2,6 dichlorophenolindophenol probably by virtue of the presence of ferrocyanide as an impurity. In the collection of blood for ascorbic acid determinations hemolysis must be avoided, since the release of hemoglobin from red blood cells produces a loss or destruction of the vitamin C of the plasma. The loss of vitamin C resulting from hemolysis is not prevented by cyanide.

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# THE TREATMENT OF ACUTE ATTACKS OF BRONCHIAL ASTHMA BY INTRAVENOUS INJECTION OF AMINOPHYLLIN\*

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THE first example of the use of purine bodies for relief of asthma was the clinical observation that hot coffee would sometimes relieve an acute attack. This was known for years, and was noted by Sollmann<sup>1</sup> who states that the mechanism was probably "partly psychic and partly bronchial." As he points out, Pal<sup>2</sup> in 1912 noted that in guinea pigs, caffeine would relax bronchial spasm induced by peptone or muscarine; also Higgins and Means<sup>3</sup> in 1915 demonstrated that caffeine would cause bronchial dilatation in the human being, and Meyer<sup>4</sup> demonstrated that it stimulates the respiratory center.

Recently other purine compounds have been found of striking benefit in the relief of asthma. As early as 1931 Herrmann and Aynesworth<sup>5</sup> successfully treated a refractory asthmatic by intravenous injection of 0.48 Gm. of aminophyllin diluted to 10 c.c. About this time also van Leeuwen<sup>6</sup> suggested the use of the xanthine group for asthma. In 1936 Hajos<sup>7</sup> noted their benefits and suggested that theophyllin facilitated the absorption of adrenalin. Efron and Tuft, in the discussion of a review by Tuft and Brodsky,<sup>8</sup> mentioned favorable results in bronchial asthma from intravenous administration of aminophyllin. Green, Paul, and Feller<sup>9</sup> in 1937 reported 16 such cases, and showed that aminophyllin caused definite increase of vital capacity, and lowered venous and intrathecal pressure without affecting arterial pressure or pulse rate. In the same month, Herrmann and Aynesworth<sup>5</sup> reported a thorough and extensive review of the subject, and presented 16 persons on whom adrenalin had been ineffectual. These received 41 injections, 31 of which "afforded prompt, complete, and persistent relief." Two cases were unsuccessful. Mild reactions to the drug were common. They noted that intravenous administration of aminophyllin apparently restored sensitivity to adrenalin. An additional case has recently been reported by Brown<sup>9</sup> who believe that adrenalin reactivity may be so restored. No fatalities have been reported.

## DATA

During a ten-month period from July 1, 1938, to May 1, 1939, 41 different patients with acute bronchial asthma were treated on the wards of the Fourth Medical Division of Bellevue Hospital. Of these 22 were found to be refractory to adrenalin, and their acute attacks were treated by intravenous injections of aminophyllin. The adrenalin used was a solution containing one part of adrenalin (as adrenalin chloride) in a thousand parts of physiologic solution of sodium chloride. It was administered subcutaneously in the usual manner. The

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TABLE I  
RESULTS IN ALL 22 PATIENTS

CASE	AGE	SEX	DISEASE DURATION	NUMBER OF INJECTIONS	RESPONSE TO 0.48 GM. AMINOPHYLLIN INTRAVENOUSLY	DURATION OF BENEFIT	TOXIC EFFECTS
1	38	M	4 years	4	Immediate	12-15 hours	Vomited
2	47	M	1 year	5	5 minutes	5-12 hours	Nausea
3	54	M	5 years	1	20 minutes	2 hours	None
4	63	M	7 years	1	15 minutes	2½ hours	None
5	28	F	5 years	2	Immediate	3-4 hours	None
6	33	F	16 years	1	Immediate	12+ hours	None
7	42	F	9 years	1	10 minutes	12+ hours	None
8	23	F	4 years	2	5 minutes	19-20 hours	Burning in chest and head
9	47	M	15 years	1	10 minutes	Slight 1 hour	None
10	21	F	3 years	35	5-30 minutes	Transient 5 minutes to 12 hours	Occasional nausea and vomiting
11	34	M	1 year	3	Immediate	4-8 hours	None
12	26	M	6 years	1	2 minutes	5 hours	Dizziness, retching and "pounding head"
13	39	M	9 years	3	5-10 minutes	3-10 hours	None
14	53	F	1 year	1	Immediate	3 hours	Vomiting
15	26	F	3 years	3	Immediate	1-2 hours	None
16	68	F	20 years	1	Immediate	No recurrence	None
17	43	F	5 years	2	Immediate	12-16 hours	Nausea and vomiting
18	29	F	Several	1	Immediate	No recurrence	Head and chest pains
19	41	F	8 years	1	10 minutes	No recurrence	None
20	36	M	Several	1	Immediate	No recurrence	None
21	73	F	Several	1	5 minutes	No recurrence	None
22	43	M	19 years	7	Immediate	12-36 hours	None

dosage, except where otherwise noted in the tables, was 0.5 c.c. No allergic studies were made on these cases. They were considered acute emergencies and treated as such. In all cases the aminophyllin was given undiluted, 0.48 Gm. in 2 c.c. of aqueous solution. The entire amount was administered intravenously within two minutes, a 25 gauge hypodermic needle preferably being used. About a third of our cases showed one or more of the following undesirable symptoms: dizziness, nausea, retching, vomiting, "pounding of the head," "burning in chest and head," or head and chest pain. No alarming effects were noted. No striking or permanent effects were noted in either pulse or blood pressure.

#### RESULTS

Twenty-two different patients were given a total of 78 injections of 0.48 Gm. or 2 c.c. of aminophyllin intravenously. No regard was given to body weight, age, or sex. Weights varied between 85 and 160 pounds, and ages from 21 to 73 years. Ten patients were males; 12 were females. The duration of disease in these patients varied from one to twenty years.

In half of the cases relief was immediate. The effect in others was evident in twenty to thirty minutes. The duration of benefit was from one hour to absence of recurrence over a period of days. In no case was it necessary to

give more than one injection within a period of less than two hours. These observations are shown in Table I.

We did not note any restoration of effectiveness of adrenalin, but can only conjecture that this may have happened in view of the number of persons that required only one injection, were subsequently discharged, and apparently did not have another refractory attack. Slightly more than one-half of our patients fall into this class.

TABLE II  
PATIENTS RECEIVING INTRAVENOUS AMINOPHYLLIN ONLY

CASE	AGE	SEX	RESPONSE	NO OF ATTACKS TREATED	TOXIC SYMPTOMS	DISEASE DURATION
1	34	M	Immediate	3	None	1 year
2	26	M	2 minutes	1	Dizziness, retching, "pounding head"	6 years
3	39	M	5-10 minutes	3	None	9 years
4	53	F	Immediate	1	Vomited	1 year
5	26	F	Immediate	3	None	3 years
6	68	F	Immediate	1	None	20 years
7	43	F	Immediate	2	Nausea and vomiting	5 years
8	29	F	Immediate	1	Head and chest pains	Several years
9	41	F	10 minutes	1	None	8 years
10	36	M	Immediate	1	None	Several years
11	73	F	5 minutes	1	None	Several years
12	43	M	Immediate	7	None	19 years

Those patients receiving intravenous injections of aqueous solution of aminophyllin for acute asthma without history of previous medication for the immediate attack are shown in Table II. In 8 of the 12 cases relief was immediate and striking. Four obtained relief within ten minutes. One-third had minor toxic symptoms. All stated that they experienced greater relief than that given by adrenalin on previous acute episodes.

TABLE III  
PATIENTS RECEIVING INTRAVENOUS AMINOPHYLLIN FOLLOWING ADRENALIN

CASE	AGE	SEX	ADRENALIN 1:1,000 SUBCUTANEOUSLY	INTERVAL FROM LAST ADRENALIN	RESPONSE TO 0.48 GM. AMINOPHYLLIN INTRAVENOUSLY	TOXIC SYMPTOMS	DISEASE DURATION
1	38	M	0.5 c.c.	24 hours	Immediate	Vomited	4 years
2	47	M	0.5 c.c. (intravenously)	45 minutes	5 minutes	Nausea	1 year
3	54	M	1.25 c.c.	25 minutes	20 minutes	None	5 years
4	63	M	0.5 c.c.	105 minutes	15 minutes	None	7 years
5	28	F	0.5 c.c.	6 1/2 hours	Immediate	None	3 years
6	33	F	0.5 c.c.	12 hours	Immediate	None	16 years
7	42	F	0.5 c.c.	8 hours	10 minutes	None	9 years
8	23	F	0.5 c.c. 8 times	40 minutes	5 minutes	Burning in chest and head	4 years

In Table III are presented 8 patients who had been given adrenalin injections by the ambulance surgeon or admitting physician for their present attack

without relief. The interval between the adrenalin and the aminophyllin injections varied from twenty-five minutes to twenty-four hours. All patients were benefited. Three of them obtained good results immediately; the others, in twenty to fifty minutes. Three had minor toxic symptoms.

TABLE IV  
ATYPICAL CASES

CASE	AGE	SEX	RESPONSE TO 0.48 GM. AMINOPHYLLIN INTRAVENOUSLY	NO. OF ATTACKS TREATED	TOXIC SYMPTOMS	REMARKS
1	47	M	10 minutes, slight	1	None	Response not striking
2	21	F	Varies from 5 minutes to half hour	35	Occasional nausea and vomiting	Originally effective but relief now only transient

The two patients in Table IV are classed by themselves. The first was a 47-year-old white man whose history was of fifteen years' standing. His benefit was only slight after ten minutes, and lasted only one hour. His clinical picture was complicated by marked emphysema and syphilitic heart disease. His asthma was of an allergic type.

The second case was that of a 21-year-old white woman who had been under almost constant treatment for the previous three years. Having been found allergic to many substances, she was not benefited by desensitization, removal of recurrent nasal polyps, or various other nose and throat procedures. Even irradiation of her sinuses, hilar regions, and blocking of her stellate ganglia offered no relief. Being accustomed to giving herself adrenalin (as much as twenty 0.5 c.c. injections of 1:1,000 solution per day), she became refractory to it. Amyl nitrite inhalations and nitroglycerin sublingually afforded only very transient relief. Emaciation and emphysema were marked. When she first received intravenous aminophyllin, nine months ago, the relief was striking and lasted several days. In the course of her disease she received a total of 35 injections. While they still afforded her some benefit, it was more transient. A complicating factor was the fact that she had few available veins, and subsequent injections had to be intramuscular; they were painful and definitely less effective.

#### SUMMARY

1. Perusal of the literature reveals that during the past nine years acute asthmatic attacks have been successfully treated by intravenous injections of aminophyllin. At least 33 cases are reported in the American literature with no fatalities.

2. During a ten-month period this drug has been extensively used for such cases on the Fourth Medical Division of Bellevue Hospital with very favorable and immediate results.

3. Twenty-two persons with severe bronchial asthma received a total of 78 injections of aminophyllin. Twenty gave almost immediate favorable response. Two responded less favorably. Eight, or 36 per cent, were long-standing asthmatics who had been treated with the usual adrenalin injections,

but whose attacks persisted unabated. Twelve persons received aminophyllin alone for their present attack with almost immediate favorable response.

4. All cases were to varying degrees refractory to adrenalin.

5. Eight cases showed minor toxic symptoms. No untoward effects were noted.

#### CONCLUSIONS

1. Aminophyllin, when given intravenously, is a most efficacious drug in the treatment of intractable acute attacks of bronchial asthma.

2. It is especially useful in cases which have become refractory to adrenalin.

NOTE: Since acceptance of our paper, the subject has also been considered by the following authors, with no untoward results noted:

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I am indebted to Dr. Charles H. Nammack for permitting the publication of this study. I wish to thank him, Dr. Gertrude H. B. Nicholson, and Dr. Arthur H. Tiber for their helpful suggestions and criticisms.

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# LABORATORY METHODS

## A NOTE ON THE DETERMINATION OF TOTAL SERUM PROTEINS, SERUM ALBUMIN, AND SERUM GLOBULIN\*

IDA KRAUS, PH.D., CHICAGO, ILL.

THE following is a modified procedure adapted from the methods of Campbell<sup>1</sup> and Koch.<sup>2</sup> Campbell's use of sodium sulfite at room temperature to precipitate serum globulins instead of sodium sulfate at 37° C. (the method of Howe<sup>3</sup>) is particularly valuable for the clinical laboratory where the facilities are limited. We† have tried the two methods in duplicate on 25 different human sera, and found that the differences between the two methods were well within the experimental error of the duplicate determinations of each method.

The procedure is now employed at the Cook County Hospital. The total nitrogen is determined in a diluted serum; the nonprotein nitrogen, in a trichloroacetic acid filtrate; the albumins (plus N.P.N.), in the filtrate from the globulins precipitated by sodium sulfite. From these data the total serum proteins, serum albumins, and globulins are calculated.

The reagents used include:

1. Sodium chloride 0.9 per cent.
2. Sodium sulfite 21 per cent.
3. Trichloroacetic acid 5 per cent.
4. Sulfuric acid 1:1.
5. Hydrogen peroxide, 30 per cent reagent grade.
6. Nessler-Folin reagent (Koch modification).
7. Standard ammonium sulfate solution (5 c.c. = 0.15 mg. nitrogen).

### PROCEDURE

*Total serum proteins* (albumins plus globulins): Pipette accurately 0.5 c.c. of serum into a 25 c.c. graduate cylinder or volumetric flask and make up to volume with 0.9 per cent of sodium chloride. Mix well by inversion and transfer 1 c.c. of the solution to a 1 by 8 inch pyrex test tube for a micro-Kjeldahl determination.

*Albumins*: Pipette 0.5 c.c. of serum into a test tube and add from a burette or pipette 9.5 c.c. of 21 per cent sodium sulfite. Mix by inversion and allow to stand at room temperature for ten minutes or longer. Filter through a twice-folded 7 cm. Whatman No. 40 filter paper. At first the solution comes through turbid, but by refiltering a water clear filtrate is obtained (if necessary add a knife edge of pumice). Transfer 0.5 c.c. of the *clear filtrate* to a 1 by 8 inch pyrex test tube for a micro-Kjeldahl determination.

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†Dr. Homer Weir and myself.

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**Nonprotein Nitrogen:** Pipette accurately 1 c.c. of serum into a 1 by 8 inch test tube or a 50 c.c. Erlenmeyer flask and measure accurately from a burette 14 c.c. of 5 per cent trichloroacetic acid. Mix well and filter. Use 4.5 c.c. of the clear filtrate for a micro-Kjeldahl determination.

**Micro-Kjeldahl:** To each tube add 1 c.c. of 1:1 sulfuric acid and a bead, and evaporate off the water by heating on a sand bath until dense white fumes of sulfuric acid fill the tube. Remove the tube and cool for thirty seconds. Then add 1 or 2 drops of 30 per cent hydrogen peroxide. Heat over the free flame for one minute. Usually the fluid remains water clear, but in case a discoloration appears, repeat the treatment with hydrogen peroxide and reheat for one minute. Treat blanks on all reagents similarly. Allow the tubes to cool and add 34 c.c. of distilled water. Stopper and mix by inversion, add 15 c.c. of Nessler-Folin reagent. Mix, let stand five minutes or more, and compare in colorimeter with the standard. The most convenient standard was found to be 5 c.c. ammonium sulfate\* (equals 0.15 mg. N) plus 1 c.c. of 1:1 sulfuric acid, and 29 c.c. water. Mix and add 15 c.c. Nessler's reagent.

**Calculations.—**

$$\text{a. Total nitrogen: } \frac{\text{Standard reading}}{\text{Unknown reading}} \times \text{Conc. standard (0.15 mg.)} \times \frac{25}{1.0} \times \frac{100}{0.5} = \text{Mg. N in 100 c.c. of serum.}$$

$$\text{b. Albumin plus N.P.N.: } \frac{S}{U} \times \text{Conc. standard} \times \frac{10}{0.5} \times \frac{100}{0.5} = \text{Mg. albumin plus N.P.N./100 c.c. serum.}$$

$$\text{c. N.P.N.: } \frac{S}{U} \times \text{Conc. standard} \times 4.5 \times \frac{100}{1} = \text{N.P.N./100 c.c. serum.}$$

(If 50 or less, the correction is insignificant and can be omitted.)

Changing N to % of protein:

$$(a - c) \times \frac{6.25}{1000} = \% \text{ total protein.}$$

$$(b - c) \times \frac{6.25}{1000} = \% \text{ albumin.}$$

$$\% \text{ total protein} - \% \text{ albumin} = \% \text{ globulin.}$$

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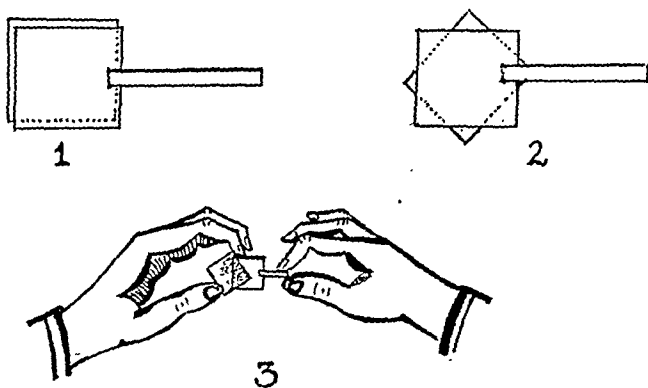
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\*If too dark or too light, make up another standard using more or less ammonium sulfate and more or less water, the other additions remaining the same.

## A SIMPLE AID IN MAKING A BLOOD SMEAR

BERNARD SILLS, MEXICO, D. F.

WHEN making blood smears with the "Ehrlich two-cover glass method" one encounters discouraging difficulties that compel one either to abandon the method and resort to one less reliable, or to devote a special amount of time to acquiring the technique. I have found that by using a simple device many of the difficulties can be overcome. The hands need not come in contact with the cleaned cover glasses until after the drop of blood has spread between their surfaces. The body of the familiar wax match used in Mexico serves satisfactorily for the wax stick.



Figs. 1-3.

One places two perfectly clean cover glasses face to face, so that their working surfaces are in contact, and then gently warms the tip of a wax stick until the wax just begins to melt. (This is done by passing the stick once or twice above the flame of a match.) The tip is then placed in contact with the edge of the top cover glass and held in place until the wax hardens (about 15 seconds), thus fixing the wax stick to the top cover glass (Fig. 1).

A drop of blood is obtained by pricking the finger or lobe of the ear. The top cover glass is lifted by means of the newly attached handle, and is touched to the drop of blood. It is then placed upon the other cover glass in such a manner that none of the corners coincide (Fig. 2). Contact causes the drop of blood to spread. Before the blood coagulates, the adherent cover glasses are lifted by the wax handle and are pulled apart by grasping the outer corners of the under glass with one hand and the wax handle with the other (Fig. 3). After drying, a gentle twist will detach the wax stick.



# AN EVALUATION OF METHODS FOR DETERMINING BLOOD AND URINARY AMYLASE\*

DANIEL L. DOZZI, M.D., PHILADELPHIA, PA.

EVANS<sup>1</sup> (1912), Fearon<sup>2</sup> (1918), Elman and McCaughan<sup>3</sup> (1927), Myers and Reid<sup>4</sup> (1933), Pickford and Dorris<sup>5</sup> (1934), Somogyi<sup>6</sup> (1938), and others have made comparisons of the various methods employed for the quantitative estimation of amylase in blood and urine. Despite these investigations, there is no uniformity of opinion regarding which method or methods should be adopted for general use. The present study was undertaken in an attempt (a) to evaluate the methods from the standpoint of adequacy; (b) to observe the limitations of and the objections to each method; (c) to determine whether the same method is adaptable for the study of both blood and urine.

## METHODS

Three basic methods are generally employed for the quantitative estimation of amylase in blood and urine. The method proposed by Wohlgemuth,<sup>7</sup> frequently called the iodine method, is based upon the disappearance of the iodine-starch blue color as the enzyme is producing hydrolysis of the starch. The second method, referred to as the saccharogenic method, has many modifications. It depends upon the determination of the amount of copper-reducing sugar formed by the enzymatic breakdown of starch. The third method, known as the viscosimetric method, relies upon the diminution in viscosity of a starch-enzyme mixture as scission of the starch particles is taking place.

The iodine method selected in the present study for both blood and urine was the Somogyi<sup>6</sup> modification of the Wohlgemuth test. Somogyi<sup>6</sup> has shown that the efficiency and accuracy of the method are increased by adopting time as the object of measurement. For the saccharogenic method, the procedure outlined by Somogyi,<sup>8</sup> which involves the Shaffer-Hartman copper-reduction method, was followed in estimating the amount of amylase in serum. The method I had adopted in another study<sup>9</sup> was used for determining the urinary amylase. The method involves the reduction of copper after the urine had been freed from interfering nitrogenous substances. The viscosimetric method, as employed by Elman and McCaughan,<sup>3</sup> was used for both urine and blood.

## PROCEDURE

Healthy female dogs were selected, placed in metabolism cages, and maintained on the same diet throughout the experiment. Twenty-four-hour collections of urine were made by catheterizing at the beginning and at the end of the twenty-four-hour period. The urine obtained by the catheterization at

\*From the Harrison Department of Surgical Research and the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia.

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the end of twenty-four hours was added to that collected from the metabolism cage. Blood samples were obtained by puncture of the jugular vein. In conjunction with other experiments, the results of which will be presented in another report, the opportunity was afforded to evaluate these methods during the normal fluctuations of amylase in the healthy dog and in dogs submitted to pancreatectomy, ligation of pancreatic ducts, administration of chloroform, and following renal injury produced by the administration of uranium nitrate.

In order that the different methods would be subjected to exactly the same conditions, the different tests were run simultaneously on both serum and urine in the same water bath at a temperature of  $40^{\circ}\text{C.} \pm 0.5^{\circ}\text{C.}$  The duration of incubation varied with the particular method, employing the same starch, electrolyte, and phosphate solutions.

A soluble potato starch was prepared according to the method of Small<sup>10</sup> and buffered to a pH of 6.8 with a phosphate solution; 5.84 Gm. of sodium chloride per 1,000 c.c. of prepared starch solution were then added. The strength of the starch solution differed with each method as follows: iodine method (0.4 per cent), saccharogenic method (2 per cent), and the viscosimetric method (7 per cent).

The amount of urine and serum used in each test also varied with the particular method in the following manner: iodine method, 1 c.c. serum, 4 c.c. urine; saccharogenic method, 0.5 c.c. serum, 5 c.c. urine; viscosimetric method, 0.4 c.c. serum, 2 c.c. urine. On the whole, each method studied was performed as advocated by the proponent of the particular method, with the exception of the starch solution and the quantity of enzyme solution used. Somogyi<sup>6</sup> used corn or rice starch in preference to potato starch. I have found potato starch entirely satisfactory. The quantities of enzyme solution were reduced because the present studies were conducted on dogs, and it is known that more amylase is found in the blood or urine of the dog than in man.

## RESULTS

In general, all the methods studied were adequate for detecting a major increase or decrease in the amount of amylase in either blood or urine. However, in certain conditions, which will be pointed out later, one method proved to be more accurate than another. When studying the amylase content of serum, the methods tended in general to parallel one another qualitatively, but there is considerable quantitative difference. A typical example of the quantitative variation is shown in Fig. 1. The curves in Fig. 1 were obtained by calculating the amount of amylase per 100 c.c. of serum and expressing it in terms of units of amylase, or milligrams of glucose according to the particular method, then plotting the per cent rise or drop of amylase following certain procedures, using the first determination for the base line. The zero point on Fig. 1, therefore, does not indicate the absence of amylase. The per cent quantitative variation is the most marked for the Wohlgemuth method and the least marked for the viscosimetric method. The variation in the quantitative expression for each method may support the theory advanced by Crandall,<sup>11</sup> that the enzyme which produces changes in viscosity may be unlike the enzyme which promotes the

formation of copper-reducing sugar from the hydrolysis of starch. Furthermore, this may substantiate the view taken by Fearon,<sup>2</sup> that each method depends upon different phenomena. Despite the possibility that one may be studying different enzymes, it would appear that when studying urine an iodine method would always display greater fluctuations. On the other hand, since the duration of incubation and the quantity of urine and serum used with each test varied with the method, it is likely that some of the quantitative differences merely indicates that one method represents the optimum of enzyme activity while another is carried further to the point where the curve of enzyme activity begins to level off.

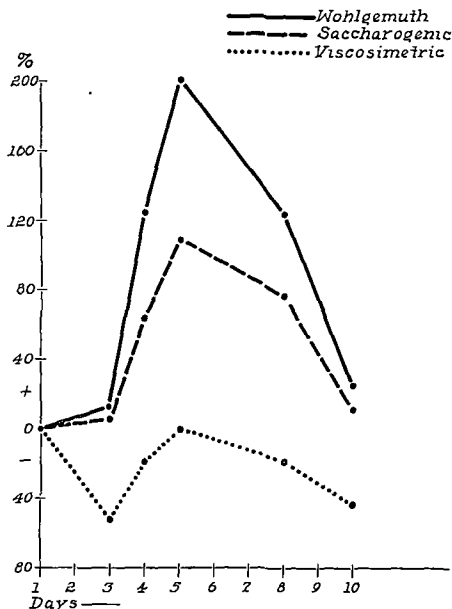


Fig. 1—Comparison of the methods used when studying serum.

The saccharogenic method was entirely satisfactory, except when studying the dogs with diabetes, in which case it was necessary to resort to smaller quantities of serum. If there is marked hyperglycemia, one has either to use great dilutions or to resort to some procedure which will eliminate the excess sugar. In either event the method would not be desirable. In general, the saccharogenic method is more accurate in that it eliminates the difficulty of detecting small color changes and is entirely applicable even if the serum is discolored by bilirubin or some other pigment. Also it excludes the possibility of decolorization of the iodine in cases with azotemia.

The iodine method was quite satisfactory, except in the icteric dogs, at which time it was impossible to interpret the color changes. Occasionally with any sample, it was difficult to obtain a clear-cut end point. The viscosimetric method was satisfactory from the standpoint of simplicity and ease of performance. However, a serious objection was noted in that oftentimes an immediate, appreciable drop in viscosity was recorded when the starch solution and serum were brought together, which was independent of enzyme action, for the same result was obtained after the serum had been heated sufficiently to destroy the enzyme. Somogyi<sup>6</sup> has recorded this same source of error and has taken pains to demonstrate that the phenomenon is independent of enzyme action.

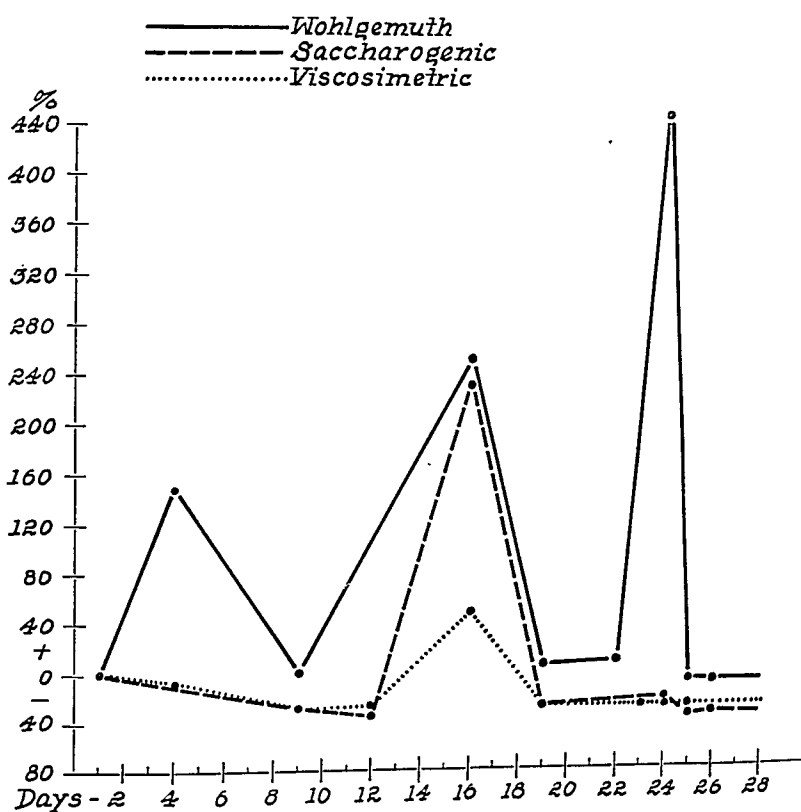


Fig. 2.—Comparison of the methods used when studying urine.

Elman and McCaughan<sup>3</sup> have also noted this error, but felt that the error was not great enough to make the method objectionable. Elman and McCaughan<sup>3</sup> have objected to the iodine and saccharogenic methods on the grounds that “a long chain of separate reactions occurs in the diastatic formation of sugar from starch, and the relative activity of the enzyme toward the different parts of the chain remains unknown” when selecting an arbitrary period of time for measurement. This objection is overcome by the modification of the iodine method used for this study wherein the progress of the amylase activity is observed at frequent intervals. One could easily do the same with the saccharogenic method by setting up a series of tubes and allowing each to incubate for different periods of time thereby determining the amylase activity for any

desired interval rather than adopting an arbitrary period of time. However, this does not seem necessary.

What has been said in the foregoing discussion concerning the methods when used for studying serum, applies, to some extent, when used with urine. The methods usually parallel one another qualitatively, as they did with serum. However, occasionally the iodine method did not follow the other two methods. A typical example of this deviation is shown for twenty-four-hour urines in Fig. 2, where it will be noted that the iodine method may indicate the presence of a large amount of amylase, whereas the other two methods show a low amylase content. This, undoubtedly, is due to decolorization of the iodine by the urine. Many workers have pointed out that all urines are capable of decolorizing iodine to a certain extent and consequently the iodine method is very unreliable with urine. The viscosimetric method is also unsuitable with urine because the initial reading following the addition of urine is practically always materially altered, necessitating frequent corrections of the "zero point." The saccharogenic method, although time-consuming, is entirely satisfactory with the same exception found when studying serum, in that it is difficult to employ if there are large quantities of sugar in the urine. While greater errors were noted when studying urine, the urine studies yielded no information that could not be obtained from the serum studies.

#### SUMMARY AND CONCLUSIONS

Iodine, saccharogenic, and viscosimetric methods were compared in both blood and urine of dogs that had had various procedures performed for producing rises and falls in amylase.

With all these methods one could detect in general appreciable changes in amylase content of blood or urine. However, there were considerable variations in the results. I conclude that while no one method is entirely satisfactory under all circumstances, the saccharogenic method is the most accurate and has fewer objectionable features than any other method studied. This method, though generally satisfactory, is difficult to employ when there is marked hyperglycemia or glycosuria. The method is equally applicable for studying blood or urine. The Wohlgemuth method is satisfactory for studying serum but is unreliable for studying urine. The viscosimetric method, being attractive for its simplicity and ease of performance, is suitable for studying serum except for an objection which has been pointed out. This method is not satisfactory for studying urine because of the necessity of frequent corrections of the "zero point."

Our observations confirm the opinions of Evans,<sup>1</sup> Fearon,<sup>2</sup> Myers and Reid,<sup>4</sup> and Somogyi<sup>6</sup> in that the copper-reduction method for estimating amylase is the most accurate.

Since all methods are less satisfactory with urine and urine yields no additional information, we question the advisability of performing routine urine studies. In order to have accurate data, the tests on urine must be performed on twenty-four-hour collections as was pointed out in another study,<sup>9</sup> a factor which introduces many difficulties. I studied<sup>9</sup> the normal fluctuations in urinary amylase in the healthy human being and found that the fluctuation

from individual to individual and in the same individual from day to day was so great that the interpretation of results is difficult. It is my belief that discrepancies in reports from different laboratories are due to normally wide ranges rather than to an inadequacy in methods for estimating amylase.

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## THE RAPID DIAGNOSIS OF MALARIA FROM THICK BLOOD SMEARS\*

GUILLERMO CASTRO, D. Sc., PANAMA, R. P.

**I**N THE tropical divisions of the United Fruit Company, malaria is the most prevalent disease. Many cases presenting the clinical symptoms of this disease appear at the hospital out-patient clinic daily, and a rapid method of blood examination is essential. A quick staining method for thick blood films, which may be completed in seven minutes or less, is described herein. Prior to the development of this method, dispensary patients suspected of having malaria were given treatment for it, and told to return the following day for further instruction after ascertaining the results of their blood examination. The old method of preparing, staining, and examining the thick films required about two hours. Under the present method, patients need wait only a few minutes for reports on their bloods.

The latest complete publication on malaria which has come to our attention is that of Nocht and Mayer,<sup>1</sup> published in 1937. Concerning thick smears, the authors state on page 127: "All preparations must be allowed to dry completely . . . before further treatment . . . they must not be used for half an hour." On page 129: ". . . stain the unfixed, well dried preparation for half an hour with Giemsa's stain as described . . . slight warming or the use of a fan ventilator accelerates drying."

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\*From the Chiriqui Land Company Hospital, Puerto Armuelles, Panama.  
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From the foregoing quotation, it is obvious that more than an hour would elapse before the preparation would be ready for examination. The method recommended here is a modification of the one mentioned by Barber and Komp<sup>2</sup> as a "quick staining of thick films for early diagnosis." It gives us reliable results in so short a time that it has been adopted as routine in our laboratory.

#### PROCEDURE

1. Place a large drop of blood (about three times the amount needed for a thin film)  $\frac{1}{4}$  inch from the end of a clean slide, and smear it well in a circle of about  $\frac{1}{2}$  inch in diameter. Place the slide on a hot plate, at about 50°-60° C., where the blood will dry in less than one minute. The top of the chalet form microscope lamp makes an excellent hot plate for this purpose. Make a semicircle with a wax pencil, enclosing the blood between the pencil mark and the edge of the slide.

2. Prepare the staining solution in the following manner: To 3 drops of old distilled water in a short tube add 1 or 2 drops of Giemsa's stain. The 4 or 5 drops of this fresh staining solution is just sufficient to cover the area enclosing the blood.

3. As soon as the blood is dry, stain for three to five minutes, with the slide in a horizontal position. Then wash very gently in a washing tray filled with tap water or, preferably, old distilled water, submerging the slide horizontally in the washing tray, moving it backwards and forwards, and removing it slowly. This will prevent the blood film from being washed away. Drain off the excess water, and stand the slide on end in an inclined and almost vertical position, on the hot plate or in front of the microscope lamp.

The slide should be removed from the hot plate the moment the smear begins to lose its moist appearance. Further heating at this point would cause decolorization. Failures are due to overheating while drying after staining. Restained smears are less satisfactory.

Our Giemsa's stain is prepared according to the directions given by Benavides:<sup>3</sup> "Place 2.4 gr. of Azur II eosin (Grubler) in a clean Erlenmeyer flask and then add 200 c.c. of C.P. anhydrous glycerin. The flask is covered with a well-fitted cork and then placed in a water-bath at 60 degrees C. for 30 minutes. The flask is shaken once or twice during this period. It is then removed from the bath, and 200 c.c. of C.P. acetone free methyl alcohol are added; and, after mixing the contents, the flask is again placed in the hot water-bath for another half hour—shaking it once or twice during this period. It is then removed, and left in the incubator overnight at 37 degrees C. Next day it is passed through filter paper and stored in 200 c.c. bottles that have been thoroughly cleansed, washed with methyl alcohol, and dried before use. These bottles are well stoppered and kept in a dark place."

#### RESULTS

The result is essentially that of the usual well-stained thick blood films. The red blood cells are completely decolorized. Schüffner's dots in large tertian parasites may be brought out in the thinner parts of the smear. Malarial pigment is readily observed. The parasites stain deeper than in the usual

thick blood films; this is especially true of the cytoplasm of tertian parasites. The leucocytes are so easily classified that we often do differential counts in our smears. The basophilic granules of some erythrocytes often seen in anemias, and especially the anemia of chronic malaria, are shown well. The presence of such basophilic staining suggests chronic malaria and calls for extensive search if parasites are not found immediately.

The time interval between the taking and the staining of the blood is of paramount importance. With an increasing interval between the taking and the staining of the blood, the stain, when used with this method, loses its ability to dehemoglobinize the red blood cells. Very poor results are obtained with bloods taken more than twelve hours in advance. Moreover, the method is not practicable for large numbers of smears (e.g., as in surveys), because each slide has to be stained separately. At the most, 4 or 5 bloods may be smeared on one slide and stained at once.

For the outpatients, and for those acutely ill and requiring prompt attention, this method is of particular value, as it permits a rapid examination and estimation of the severity of infection, the quality and approximate quantity of the leucocytes, and the response to treatment. In cases in which a prompt diagnosis is not urgent, the method of Barber and Komp may be preferred.

#### SUMMARY

A rapid method for the staining of thick blood films for the diagnosis of malaria, and its applicability to routine dispensary and hospital work, is described.

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# THE DETERMINATION OF pH VALUES OF BIOLOGICAL FLUIDS

## II. THE INFLUENCE OF TEMPERATURE, DILUTION, AND THE TYPE OF DILUENT UPON THE pH VALUES OF BACTERIOLOGIC MEDIA\*

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### INTRODUCTION

THE pH values of a number of biological products and bacteriologic media, obtained by colorimetric and electrometric methods at 25° C. and 30° C., have been presented in a recent paper.<sup>1</sup> Depending upon the particular colorimetric procedure employed, the test solutions were diluted with fixed quantities of distilled water or physiologic saline solution. The pH values of the undiluted test fluids, obtained with the glass electrode, were in agreement well within the limits  $\pm 0.3$  pH unit, with the values of the diluted test fluids obtained by means of the different colorimetric methods. In the present work, the pH values of a number of buffer solutions and bacteriologic media, diluted with varying quantities of distilled water or saline solution, have been determined at temperatures between 10° C. and 38° C.

In order to determine the pH values of the bacteriologic media as accurately as possible at the stated temperatures, a series of pH determinations was first made on standard buffer mixtures. These data were then tested for their degree of conformity with thermodynamic principles and an approximate form of the Debye-Hückel limiting law. A comparison was also made with the pH values of these reference buffers obtained by MacInnes and Hitchcock and their co-workers at the various temperatures.<sup>2-4</sup> From these tests it was found that the MacInnes-Belcher glass electrode, in conjunction with the Young Electron Ray Meter, give accurate and reproducible results at the temperatures of measurement.

The general problem of pH measurement, as well as the specific applications of pH measurements to problems in bacteriology, has been fully described by Clark.<sup>5, 6</sup> Zimmermann has recently made a study of the errors involved in electrometric pH determinations of culture media.<sup>7</sup> Employing the quinhydrone electrode and saturated calomel electrode system, he studied the variation of pH with temperature for bouillon and agar bouillon media. He also measured the effect of aqueous dilution upon the pH values of the media.

The use of semi-fluid media for bacterial cultivation has presented a new problem in pH determination. It has been generally recognized that colorimetric methods do not always give satisfactory and accurate results.<sup>8</sup> To this end, the present study of bacteriologic media has been extended to include pH measure-

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ments at various temperatures on a semi-fluid medium and on a semi-solid medium commonly employed in bacterial culture.

#### EXPERIMENTAL METHODS

The procedure adopted in this work consisted of the measurement of pH values of the test solutions set up in a galvanic cell with liquid junction:

Glass, Test Solution : KCl (saturated) : HgCl, Hg

The Young Electron Ray Meter was employed in conjunction with a MacInnes-Belcher durable condenser type glass electrode. The latter has been fully described in the literature.<sup>9, 10</sup> The Young Electron Ray Meter is a potentiometer capable of recording either in millivolts or pH units. In these experiments, the instrument was set to read directly in pH units. The Electron Ray Meter employs a cathode ray tube in conjunction with a five-element vacuum tube as null-point indicator.

Buffer solutions were prepared with Baker's "analyzed" salts. A 0.05 molar primary potassium phthalate ( $\text{KHC}_8\text{H}_4\text{O}_4$ ) solution was used as a primary pH standard. This solution was prepared from the crystalline salt purchased from the National Bureau of Standards. The pH values of 0.05 molar primary potassium phthalate have been carefully determined at various temperatures by MacInnes and his co-workers.<sup>4</sup> On the basis of their work, the following pH values were assigned to the standard phthalate solution and used throughout this work:

	0.05 M $\text{KHC}_8\text{H}_4\text{O}_4$						
t° C.	10	15	20	25	30	35	38
pH	4.00	4.00	4.00	4.00	4.01	4.02	4.02

The bacteriologic media were prepared from the best ingredients available, according to the methods described mainly by Wadsworth.<sup>11</sup> Suitable precautions were observed in order to maintain the sterility of the test solutions. Dilutions were made with sterile distilled water or physiologic saline solution, and the solutions were stored in sterile 1 ounce bottles. Immediately before and after pH determinations, the test solutions were stored in the icebox at about 7° C.

pH measurements were carried out by enclosing the glass electrode filled with test fluid in an electrically grounded metal case, which in turn was enclosed in an incubator with thermostatic temperature control. By means of the latter, the temperature around the glass electrode assembly (glass and saturated calomel electrodes) was kept constant within  $\pm 0.5^\circ$  C. during any measurement. When not in use, the glass electrode was filled with distilled water. By this practice, the residual potential within the glass membrane (asymmetry potential) was maintained at a low constant level.<sup>9</sup> pH measurements were made with sodium acetate and borate buffer mixtures in order to cover the region pH 4.0 to pH 9.0. Within this pH range, the MacInnes-Belcher glass electrode, in conjunction with the Young Electron Ray Meter, functioned accurately within the limits  $\pm 0.02$  pH unit.

#### EXPERIMENTAL RESULTS

The pH values in Tables I and II represent the results of at least three concordant measurements.

The following are the compositions of the media employed in these experiments\*:

(A) Pneumococcus broth: beef heart infusion, peptone (Parke-Davis) 1 per cent, disodium phosphate 0.2 per cent.

(B) Veal broth: veal infusion, peptone (Difco) 1 per cent, sodium chloride 0.5 per cent.

(C) Glucose beef heart phosphate broth: beef heart infusion, peptone (Parke-Davis) 2 per cent, glucose 0.5 per cent, disodium phosphate 0.2 per cent.

(E) Diphtheria broth: distilled water 7 liters, bacto-veal (Difco) 1 lb., proteose peptone 2 per cent, sodium chloride 0.5 per cent, sodium acetate 1 per cent.

(F) Hormone broth (semi-fluid): beef heart infusion, peptone 1 per cent, sodium chloride 0.5 per cent, gelatin 0.2 per cent.

(G) Glucose beef heart phosphate agar (semi-solid): beef heart infusion, peptone 1 per cent, glucose 0.03 per cent, disodium phosphate 0.2 per cent, agar 0.25 per cent.

Table I summarizes the results obtained with the acetate and borate buffer mixtures. The changes in the pH values for any one solution of the acetate buffer mixtures was 0.07 pH unit or less between 10° C. and 38° C. In column (1) is given the degree of dilution or the percentage dilution of the acetate buffer mixture with distilled water or physiologic saline (0.85 per cent NaCl) solution; columns (2) and (3) show the molar concentrations of acetic acid and sodium acetate, respectively, after aqueous or saline dilution; and column (4) shows the molar concentration of sodium chloride in the buffer solutions diluted with physiologic salt solution. The maximum and minimum pH values for the dilutions between 10° C. and 38° C. for the aqueous and saline buffer mixtures are shown in columns (5) and (6), and the average pH values between 10° C. and 38° C. for all dilutions of the buffer solutions made up with distilled water and with saline solution are shown in columns (7) and (8).

For the borate buffer mixtures, columns (1) to (3) have the same significance as in the first part of Table I. The pH values of the test solutions diluted with distilled water or physiologic saline solution at the different temperatures are presented under column (4).

From the data in Table I, it is evident that for the acetate and borate buffer mixtures, the pH values increased with increasing aqueous dilution at all temperatures studied. Dilution with physiologic saline solution, on the other hand, decreased the pH values or increased the hydrogen-ion activities of the buffers. For both the diluted and undiluted borate buffer mixtures, the pH values decreased with increasing temperature, but for the acetate mixtures, the pH values at the different temperatures remained constant within the limits of precision of the instrument. These results are in agreement with those obtained by Sörenson,<sup>12</sup> Kolthoff,<sup>13</sup> and others.<sup>6</sup>

\*Thanks are due to Miss M. McGrath, of the Media Division, for kindly providing the media used in this work.

TABLE I  
 ACETIC ACID—SODIUM ACETATE BUFFERS

(1)	(2)	(3)	(4)	(5)		(6)	(7)		(8)
% DIL.	CH <sub>3</sub> COOH	CH <sub>3</sub> COONa	NaCl	RANGE OF pH VALUES 10° C.—38° C.		AVERAGE pH VALUES 10° C.—38° C.			
				H <sub>2</sub> O DILUTION	0.85% NaCl DILUTION		H <sub>2</sub> O DILUTION	0.85% NaCl DILUTION	
0	0.05230	0.05000		4.62—4.68			4.65		
10	0.04707	0.04500		4.62—4.68			4.66		
25	0.03923	0.03750	0.01454	4.63—4.69	4.63—4.69		4.67	4.66	
50	0.02615	0.02500	0.03635	4.63—4.70	4.62—4.68		4.67	4.65	
75	0.01308	0.01250	0.07271	4.67—4.72	4.59—4.65		4.70	4.62	
90	0.00523	0.00500	0.10906	4.74—4.77	4.58—4.64		4.76	4.61	
			0.13087		4.58—4.64			4.61	

BORAX BUFFER SOLUTIONS

(1)	(2)	(3)	(4)						
% DIL.	Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub>	NaCl	pH VALUES AT VARIOUS TEMPERATURES—t° C.						
			10	15	20	25	30	35	38
0	0.05000		9.30	9.26	9.21	9.18	9.09	9.06	9.05
10	0.04500		9.30	9.26	9.21	9.18	9.10	9.06	9.05
25	0.03750	0.01454		9.20	9.21	9.15	9.10	9.05	9.03
50	0.02500	0.03635	9.29	9.26	9.20	9.18	9.11	9.06	9.05
75	0.01250	0.07271	9.29	9.16	9.18	9.14	9.09	9.03	9.00
90	0.00500	0.10906	9.29	9.26	9.20	9.18	9.11	9.07	9.05
		0.13087	9.30	9.11	9.13	9.11	9.04	8.99	8.96
				9.27	9.21	9.19	9.11	9.07	9.05
				9.07	9.08	9.08	9.01	8.96	8.92
				9.22	9.23	9.21	9.13	9.09	9.09
				9.03	9.06	9.06	8.99	8.94	8.90

Table II summarizes the results obtained with the bacteriologic media. The first part of the table shows the pH data for aqueous dilutions of the media. The second part shows the results obtained with saline dilutions of the media. Column (1) gives the names and composition of the media; column (2) indicates the degree of dilution with distilled water or physiologic saline solution; column (3) shows the pH values at various temperatures of the diluted and undiluted media.

The pH data under column (3) in Table II show that the pH values increased with increasing aqueous dilution for pneumococcus broth, veal broth, and glucose beef heart phosphate broth (pH unadjusted). In the case of diphtheria broth and glucose beef heart phosphate broth (pH adjusted colorimetrically) there was a decrease with increasing aqueous dilution. The pH values of the hormone broth and glucose beef heart phosphate agar remained fairly constant (ca.  $\pm 0.03$  pH unit), with either aqueous or saline dilution to about 50 per cent dilution. The results with saline dilution were more uniform than with aqueous dilution of the media. The hydrogen-ion activities of all the media studied increased with increasing saline dilution.

Fig. 1 illustrates graphically the linear relationship between pH and temperature for the undiluted media. Empirical pH values below 10° C. and above 38° C. may be obtained by graphical extrapolation of the appropriate data for either the diluted or undiluted media.

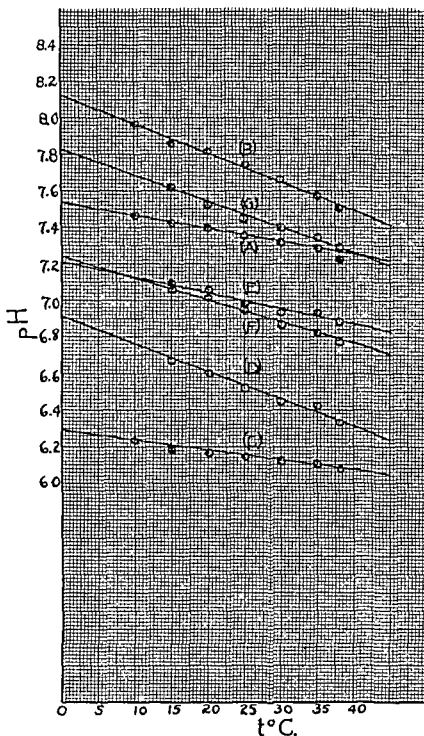


Fig. 1.—The variation of pH with temperature for the undiluted media. The letters at the end of the curves correspond to those given in Table II.

From the data at 25° C. for aqueous dilutions of glucose beef heart phosphate broth (pH unadjusted), the following linear equation has been obtained by the Method of Least Squares:

$$\frac{\text{pH}}{\text{Percentage dilution}} = 0.29 + \frac{6.14}{\text{Percentage dilution}} \quad (1)$$

By rearranging the terms in equation (1) and substituting the appropriate values for the percentage dilution, the pH values of this medium at different degrees of dilution have been computed. The average difference between the observed and calculated pH values at 25° C. was found to be  $\pm 0.03$  pH unit. All

TABLE II  
WATER DILUENT

(1) MEDIA	(2) % WATER DILUTION	(3) PH VALUES OF MEDIA AT VARIOUS TEMPERATURES—t° C.						
		10	15	20	25	30	35	38
(A) Pneumococcus broth	0	7.47	7.43	7.40	7.35	7.31	7.29	7.23
	10	7.47	7.43	7.40	7.37	7.32	7.30	7.25
	25	7.48	7.44	7.42	7.38	7.33	7.31	7.26
	50	7.51	7.46	7.45	7.42	7.37	7.34	7.29
	75	7.53	7.50	7.49	7.45	7.41	7.39	7.34
	90	7.53	7.50	7.49	7.45	7.43	7.42	7.36
(B) Veal broth	0	7.97	7.87	7.82	7.75	7.67	7.58	7.51
	10	7.97	7.87	7.83	7.75	7.68	7.59	7.51
	25	7.93	7.85	7.83	7.75	7.68	7.58	7.50
	50	7.95	7.85	7.83	7.76	7.70	7.61	7.53
	75	7.92	7.84	7.86	7.78	7.72	7.62	7.54
	90	7.77	7.77	7.83	7.74	7.69	7.62	7.53
(C) Glucose beef heart phosphate broth (pH unadjusted be- fore measurements)	0	6.24	6.19	6.17	6.15	6.13	6.11	6.08
	10	6.24	6.21	6.19	6.17	6.13	6.11	6.08
	25	6.24	6.21	6.20	6.18	6.15	6.12	6.08
	50	6.30	6.25	6.25	6.23	6.20	6.18	6.14
	75	6.37	6.34	6.33	6.32	6.29	6.25	6.22
	90	6.43	6.40	6.39	6.37	6.35	6.30	6.26
(D) Glucose beef heart phosphate broth (pH adjusted colorimetrically)	0		7.68	7.61	7.52	7.45	7.42	7.33
	10		7.67	7.60	7.51	7.45	7.42	7.33
	25		7.67	7.60	7.51	7.45	7.42	7.33
	50		7.67	7.60	7.51	7.45	7.43	7.34
	75		7.61	7.54	7.47	7.43	7.40	7.31
(E) Diphtheria broth	0		7.06	7.01	6.90	6.85	6.84	6.79
	10		7.01	7.00	6.89	6.84	6.84	6.79
	25		7.01	6.98	6.89	6.83	6.84	6.79
	50		6.98	6.98	6.87	6.82	6.82	6.78
	75		6.97	6.95	6.85	6.82	6.84	6.80
	90		6.86	6.85	6.78	6.75	6.80	6.79
(F) Hormone broth (semi-fluid)	0		7.08	7.05	6.95	6.89	6.83	6.78
	10		7.04	7.01	6.93	6.87	6.83	6.77
	25		7.04	7.02	6.93	6.87	6.85	6.78
	50		7.05	7.03	6.95	6.89	6.86	6.80
	75		7.03	7.02	6.95	6.89	6.86	6.80
	90		6.88	6.91	6.86	6.82	6.85	6.85
(G) Glucose beef heart phosphate agar (semi-solid)	0		7.62	7.53	7.45	7.40	7.35	7.29
	10		7.57	7.50	7.42	7.39	7.33	7.29
	25		7.57	7.50	7.43	7.39	7.34	7.29
	50		7.55	7.50	7.42	7.39	7.34	7.29
	75		7.43	7.38	7.31	7.31	7.25	7.25
	90		7.28	7.26	7.22	7.27	7.25	7.21

the media studied appear to obey the linear relationship between pH/percentage dilution and the reciprocal of the percentage dilution (1/percentage dilution) for aqueous and saline dilutions of the media at all temperatures of measurements. Fig. 2 shows the results obtained with aqueous dilutions of some of the media at 25° C.

## DISCUSSION

An examination of the pH data in Table I and Table II indicates that the pH values of all the test solutions decreased with increasing temperature (except for the acetate buffer mixtures). Moreover, the pH values of all test solutions decreased with increasing saline dilution at all temperatures. The results obtained for aqueous dilution were not uniform for all the media. The pH values of some of the media increased while the pH values of other media de-

TABLE II—CONT'D  
0.85 PER CENT SODIUM CHLORIDE DILUENT

(1) MEDIA	(2) % SALINE DILUTION	(3) pH VALUES OF MEDIA AT VARIOUS TEMPERATURES—t° C.						
		10	15	20	25	30	35	38
(A) Pneumococcus broth	0	7.47	7.43	7.40	7.35	7.31	7.29	7.23
	10	7.45	7.40	7.39	7.35	7.32	7.29	7.24
	25	7.45	7.40	7.38	7.35	7.32	7.29	7.24
	50	7.44	7.40	7.37	7.35	7.31	7.29	7.22
	75	7.41	7.35	7.34	7.33	7.29	7.25	7.20
	90	7.34	7.30	7.31	7.30	7.25	7.22	7.18
(B) Veal broth	0	7.97	7.87	7.82	7.75	7.67	7.58	7.51
	10	7.94	7.85	7.82	7.75	7.68	7.58	7.51
	25	7.94	7.85	7.82	7.75	7.68	7.58	7.50
	50	7.88	7.85	7.82	7.73	7.66	7.56	7.48
	75	7.84	7.75	7.75	7.68	7.61	7.53	7.45
	90	7.74	7.68	7.73	7.63	7.58	7.49	7.42
(C) Glucose beef heart phosphate broth (pH unadjusted be- fore measurements)	0	6.24	6.19	6.17	6.15	6.13	6.11	6.08
	10	6.22	6.19	6.18	6.16	6.14	6.07	6.07
	25	6.22	6.18	6.17	6.16	6.14	6.07	6.07
	50	6.22	6.16	6.17	6.16	6.14	6.07	6.07
	75	6.21	6.16	6.16	6.16	6.13	6.08	6.06
	90	6.20	6.14	6.13	6.13	6.13	6.08	6.06
(D) Glucose beef heart phosphate broth (pH adjusted colorimetrically)	0		7.68	7.61	7.52	7.45	7.42	7.33
	10		7.63	7.56	7.47	7.42	7.40	7.30
	25		7.63	7.56	7.47	7.42	7.39	7.30
	50		7.62	7.55	7.45	7.40	7.36	7.27
	75		7.55	7.48	7.39	7.34	7.32	7.23
(E) Diphtheria broth	0		7.06	7.01	6.90	6.85	6.84	6.79
	10		7.01	6.98	6.89	6.83	6.84	6.78
	25		7.00	6.97	6.88	6.83	6.83	6.78
	50		6.97	6.95	6.85	6.81	6.82	6.77
	75		6.94	6.93	6.83	6.79	6.80	6.76
	90		6.76	6.79	6.75	6.74	6.78	6.74
(F) Hormone broth (semi-fluid)	0		7.08	7.05	6.95	6.89	6.83	6.78
	10		7.03	7.00	6.91	6.85	6.82	6.77
	25		7.04	7.02	6.93	6.87	6.83	6.78
	50		6.99	6.97	6.90	6.94	6.85	6.76
	75		6.99	6.97	6.90	6.84	6.84	6.76
	90		6.85	6.85	6.79	6.72	6.67	6.57
(G) Glucose beef heart phosphate agar (semi-solid)	0		7.62	7.53	7.45	7.40	7.35	7.29
	10		7.55	7.48	7.40	7.39	7.33	7.28
	25		7.55	7.48	7.41	7.38	7.33	7.28
	50		7.54	7.48	7.41	7.38	7.32	7.27
	75		7.28	7.26	7.22	7.25	7.25	7.21
	90		7.26	7.23	7.18	7.19	7.17	7.13

creased with increasing aqueous dilution. Up to about 50 per cent dilution with distilled water or saline solution, the changes in pH values of the media were fairly small.

While nutrient media are used between 37° C. and 38° C. for the cultivation of microorganisms, the pH values of media are generally determined at room temperature between 20° C. and 25° C. Table II shows moderate but real differences in the pH values of the media between 20° C. and 38° C. which ranged up to 0.5 pH unit. This was true for the diluted and the undiluted media. These facts must, therefore, be considered in the determination of the initial optimum pH values of media for bacterial cultivation.

In view of the preceding considerations, the linear relationship developed between pH and temperature on the one hand, and pH and the degree of dilu-

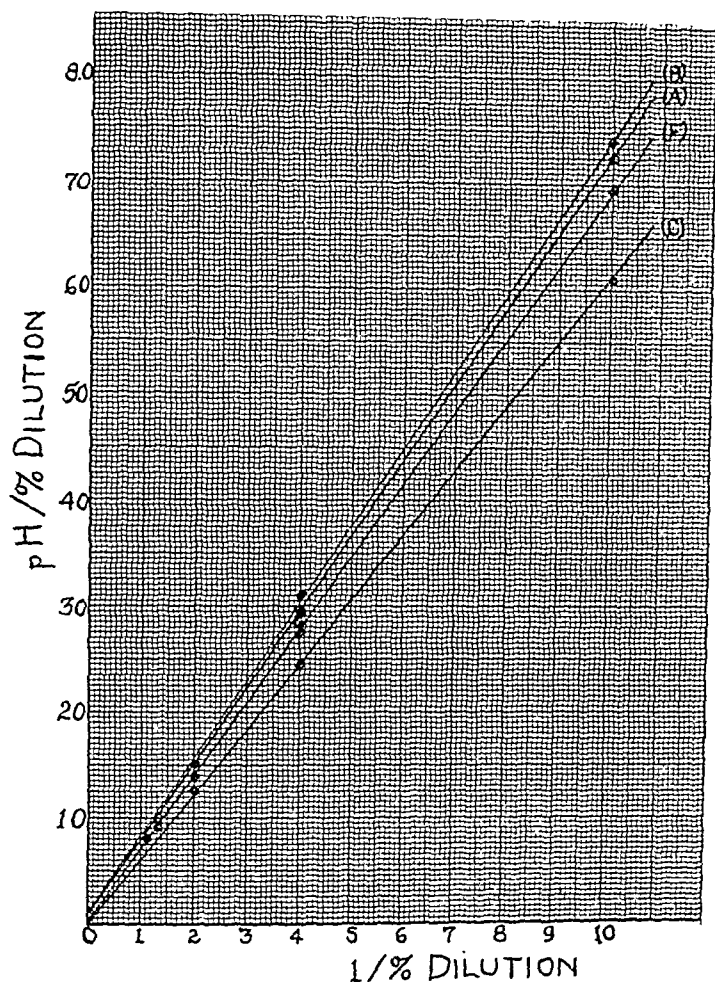


Fig. 2.—The variation of pH values of the media with the degree of aqueous dilution (per cent dilution). The letters at the end of the curves correspond to those given in Table II.

tion on the other, is useful in colorimetric determinations of the pH values of media. By the measurement of pH values of any medium at two or three temperatures, the pH values at other intermediate temperatures are predictable with a fair degree of accuracy. Although it has been shown that empirical pH values below  $10^{\circ}\text{C.}$  and above  $38^{\circ}\text{C.}$  may be obtained by extrapolation of the appropriate data, such pH values are strictly empirical and have no theoretical significance as yet. Likewise, since the dilution of test fluids is an essential procedure in the colorimetric method, the pH values of media may be determined at two or three dilutions with a specific diluent. From the relationship developed for the pH/percentage dilution and the reciprocal of the per cent dilution, pH values may be evaluated at any other dilutions with the same diluent fairly accurately.

#### SUMMARY

The pH values of a number of diluted and undiluted buffer solutions and bacteriologic media have been determined between  $10^{\circ}\text{C.}$  and  $38^{\circ}\text{C.}$  from measurements in a cell with liquid junction. Linear relationships for the media studied have been indicated between pH and temperature on the one hand, and



pH and degree of dilution on the other. The Young Electron Ray Meter, in conjunction with the MacInnes-Belcher durable condenser type glass electrode, has been found to yield accurate and reproducible pH values within the limits of experimental error, over the temperature and pH ranges studied.

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## A PROCEDURE FOR THE ROUTINE DETERMINATION OF VITAMIN B<sub>1</sub> IN URINE\*

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THE Hennessey and Cerecedo<sup>1</sup> method for determining the vitamin B<sub>1</sub> content of biological material has been modified to adapt it to the routine determination of vitamin B<sub>1</sub> in urine. A preliminary extraction with benzyl alcohol<sup>2</sup> is not necessary for the small amount of urine required in the determination of vitamin B<sub>1</sub> by the thiochrome reaction. The permutit adsorption can be regulated so that other urinary constituents do not interfere with the adsorption and elution of the vitamin. Urine samples may be run directly through simple adsorbing columns at room temperature, and a large number of determinations may be carried out simultaneously. The thiochrome reaction is not difficult to control in potassium chloride eluates from these columns, and the recovery of thiamine added to urine samples is about as good as its recovery from standard aqueous solutions (Table I).

Adsorption columns are set up with "Folin" permutit† which has been heated for two hours on a steam bath with an equal volume of 25 per cent potassium chloride kept acid to Congo red with sulfuric acid and then washed free of excess potassium by four changes of 0.1 per cent acetic acid. A column of this permutit, 1 cm. by 7 cm., is used, and the flow through it is kept between 1 and 0.5 c.c. per minute by a fine capillary at the bottom of the adsorption tube.‡ One to 10 c.c. of filtered urine made up to a total volume of 10 c.c. and acidified with acetic acid to pH 4.5 (nitrazine paper) are run through a column, and the unadsorbed urinary substances are in great part removed by washing with 20 c.c. of distilled water and 10 c.c. of hot 0.1 per cent acetic acid. The vitamin is eluted by passing over the column 15 c.c. of boiling 25 per cent potassium chloride acidified to pH 2 (Congo red) with sulfuric acid. The washing with hot acetic acid and the elution are carried out at an elevated temperature maintained by suspending the tubes in a chamber heated by an ordinary electric heat reflector. Tubes are not allowed to run dry while being heated, and fresh permutit is used with each urine sample.

Three cubic centimeters of alkaline ferrieyanide (30 c.c. of 15 per cent sodium hydroxide and 1 c.c. of 1 per cent potassium ferrieyanide) and 13 c.c. of freshly distilled isobutanol are added in the order given to a 5 c.c. aliquot of cooled eluate in a small separatory funnel. A trace of the yellow ferrieyanide color should persist in the aqueous phase for at least ten seconds. When an occasional eluate requires ferrieyanide, a drop or two of 1 per cent potassium ferrieyanide may be added directly without alteration of the thiochrome yield,

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†Permutit Co., New York City.

‡Made by E. Machlett & Sons, New York City.

TABLE I  
RECOVERY OF VITAMIN B<sub>1</sub> FROM PERMUTIT COLUMNS

DATE	TYPE OF SOLUTION	VITAMIN CONTENT IN GAMMAS	PER CENT OF ADDED VITAMIN RECOVERED
10/6	10 c.c. of aqueous solution plus 1 drop of acetic acid	2	97
	10 c.c. of urine pH 4.5	2.3	
	10 c.c. of same urine pH 4.5 plus 2 gammas of vitamin B <sub>1</sub>	4.35	103
10/11	5 c.c. of urine pH 4.5	1.68	
	10 c.c. of same urine pH 4.5	3.26	
	5 c.c. of same urine pH 4.5 plus 2 gammas of vitamin B <sub>1</sub>	3.45	90
	10 c.c. of same urine pH 4.5 plus 2 gammas of vitamin B <sub>1</sub>	5.10	90
	10 c.c. of aqueous solution plus 1 drop of acetic acid	2	87
10/27	10 c.c. of urine pH 4.5	2.57	
	10 c.c. of same urine pH 4.5	2.53	
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B <sub>1</sub>	3.45	90
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B <sub>1</sub>	3.46	91
	10 c.c. of aqueous solution plus 1 drop of acetic acid	1	95
10/28	10 c.c. of urine pH 4.5	0.71	
	10 c.c. of same urine pH 4.5	0.66	
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B <sub>1</sub>	1.60	92
	10 c.c. of same urine pH 4.5 plus 1 gamma of vitamin B <sub>1</sub>	1.61	93

TABLE II  
CONVERSION OF THIAMINE TO THIOCHROME BY ALKALINE OXIDATION AND IMMEDIATE EXTRACTION WITH ISOBUTANOL

THIAMINE IN SAMPLES, GAMMAS	THIOCHROME FLUORESCENCE IN GALVANOMETER UNITS*
0.2	10
0.5	26
1.0	50
1.0	50†
1.5	73

\*0.52 gamma of a fresh thiochrome solution which has been protected from light gives 50 galvanometer units of fluorescent light when treated with alkaline ferricyanide and extracted with isobutanol. Since the molecular weight of thiamine chloride is 335, and the molecular weight of thiochrome is 262, the values given in this table represent approximately 67 per cent conversion of thiamine to thiochrome.

†An additional 0.2 c.c. of 1 per cent potassium ferricyanide was added to this sample fifteen seconds before extraction with isobutanol was begun.

providing the thiochrome is at once shaken out into the isobutanol (Table II). The thiochrome is extracted by vigorous shaking for one and one-half minutes, the layers are separated by centrifuging for one minute, and the aqueous layer is drawn off and discarded. The isobutanol is dried over a little anhydrous sodium sulfate for a moment, and the thiochrome fluorescence is measured in a Pfaltz and Bauer photoelectric fluorometer<sup>1</sup> standardized against known thiamine solutions.\*

\*The thiochrome content of these butanol extracts can also be estimated by comparison with standard thiochrome solutions in ultraviolet light. Thiochrome for making these solutions may be obtained from Merck & Co., Rahway, N. J.

TABLE III

I. Effect on contaminating blue fluorescences in urine eluates of five-minute treatment of aliquots of these eluates with 3 c.c. of alkali in atmosphere of nitrogen.			
a. Fluorescence of immediate butanol extract	20	Fluorescence of butanol extract made at end of alkali treatment	
b. Fluorescence of butanol extract of similar aliquot treated with alkali and ferrieyanide and extracted at once	44	Fluorescence of butanol extract when ferrieyanide is added to eluate at end of five-minute treatment with alkali in nitrogen atmosphere	
c. 1 gamma of thiamine in potassium chloride treated as in "b" above	53	1 gamma of thiamine in potassium chloride treated as in "b" above	
		51	
II. Differences in the behavior of isobutanol extracts from oxidized (A) and from unoxidized (B) urine eluates. All extractions are made from solutions of comparable volume and alkalinity.			
A		B	
Fluorescence in galvanometer units	After shaking with 3 c.c. of alkaline ferrieyanide	Fluorescence in galvanometer units	After shaking one to two minutes with 3 c.c. of alkaline ferrieyanide
44	44	20	13
6	6	11	3
Similar treatment of butanol extracts from a standard solution of thiamine.			
30	30	3	11
III. Additive fluorescences obtained by extracting oxidized urine eluate with butanol previously used to extract the thiochrome obtained from 1 gamma of thiamine.			
a. Fluorescence obtained from an aliquot of urine eluate using freshly distilled butanol		b. Fluorescence obtained from 1 gamma of thiamine using freshly distilled butanol	c. Fluorescence obtained by using butanol of "b" to extract another aliquot of the same urine eluate
30		52	79*

\*Theoretically, with a reagent blank of 3 one would expect to obtain in (c) a fluorescence equal to  $(30-3) + 52$  or 79.

The conversion of thiamine to thiochrome in both standard solutions and eluates by this procedure is approximately 67 per cent of the theoretical maximum for amounts of thiamine between 0.2 and 1.5 gammas (Table II). In practice the fluorometer is so adjusted that one gamma of thiamine when converted to thiochrome gives a galvanometer deflection of 54 units when the reagent blank is equal to four units. For convenience, future adjustment of the fluorometer is made against a permanent quinine standard<sup>1</sup> prepared to give a fluorescence equal to that obtained by the 67 per cent conversion of one gamma of thiamine to thiochrome.

Blank determinations for the thiochrome method are conventionally obtained by treating an aliquot with sodium hydroxide and extracting the alkaline solution with isobutanol. Such blanks vary considerably with different urine eluates, but probably do not represent true blanks because in them the effect of oxidation upon contaminating fluorescent material is of necessity lacking. The blue fluorescence that appears in the blanks from permuted eluates is a lighter blue than that of thiochrome, is more sensitive than thiochrome to prolonged treatment with alkali or to brief treatment with alkaline potassium ferrieyanide, and for this reason, probably does not appear in the butanol extracts of oxidized eluates. As a result, blanks on urines of low vitamin B<sub>1</sub> content are often

greater than the thiochrome readings. When one of these blank butanols from an unoxidized urine eluate is shaken with alkaline ferricyanide, its light blue fluorescence disappears and is replaced by a thiochrome-like fluorescence of about the same order of magnitude as that which is obtained when a butanol blank from a standard solution of similar vitamin B<sub>1</sub> content is shaken with 3 c.c. of alkaline ferricyanide. On the other hand, the fluorescence of butanol extracts from oxidized eluates is like the fluorescence of pure thiochrome extracts

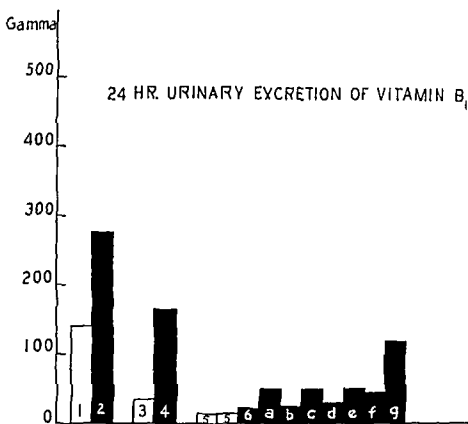


Fig. 1.—1, Average twenty-four-hour urinary excretion of vitamin B<sub>1</sub> in 10 interns. 2, Average response of 1 to 500  $\gamma$  of vitamin B<sub>1</sub> subcutaneously. 3, Average twenty-four-hour urinary excretion of vitamin B<sub>1</sub> in 5 patients with temporarily inadequate intake as a result of recent acute illness. 4, Average response of 3 to 500  $\gamma$  of vitamin B<sub>1</sub> subcutaneously. 5, Twenty-four-hour urinary excretion of vitamin B<sub>1</sub> in a patient with clinical vitamin B<sub>1</sub> deficiency. 6, a-e response of 5 to daily injections of 500  $\gamma$  of vitamin B<sub>1</sub> subcutaneously; f and g response of 5 to daily injections of 1,000  $\gamma$  of vitamin B<sub>1</sub> subcutaneously.

in that it is not altered by reshaking with 3 c.c. of alkaline ferricyanide. Butanol extracts from oxidized eluates fluoresce with what appears to be a pure thiochrome color and seem to contain no materials interfering with thiochrome fluorescence. The fluorescences obtained by extracting separate portions of oxidized urine eluate with butanol and with butanol plus a known amount of thiochrome give strictly additive readings. This is contrary to the experience of Westenbrink<sup>3</sup> with franconite eluates. As a result of these circumstances, which are illustrated in Table III, we have felt it preferable to omit determination of the blank and to substitute an arbitrary blank deduction equal to the reagent blank.

#### DISCUSSION

During the past year we have made a large number of determinations of the urinary excretion of vitamin B<sub>1</sub> using permutit adsorption columns. Recovery of thiamine added to these urines has been  $95 \pm 15$  per cent. Recently, using the procedure as above outlined, recoveries have averaged  $92 \pm 5$  per cent. The chief source of error in the method appears to lie in the eluting process

which probably could be improved by a better control of temperature and rate of flow. Since the clinical variations which appear to be diagnostically significant are considerably greater than those inherent in the method (Fig. 1) we have not felt it necessary to complicate the present procedure by further refinements. It might be mentioned that these results are more consistent than those we obtained by omitting the permittit adsorption and substituting a more time-consuming procedure similar to that recently perfected by Wang and Harris.<sup>4</sup>

Our experience with the stability of vitamin B<sub>1</sub> in urine has been similar to that of Melnich and Fields.<sup>2</sup> The vitamin B<sub>1</sub> content of urine is not altered by twelve hours' incubation even when its pH is somewhat on the alkaline side, and it appears that the twenty-four-hour urinary excretion of vitamin B<sub>1</sub> is, under most circumstances, a fairly reliable index of the patient's vitamin B<sub>1</sub> intake. However, when this excretion is abnormally low, less than 50 gammas per twenty-four hours, it is important to distinguish between cases in which this is due to a temporarily inadequate vitamin B<sub>1</sub> intake, such as may occur in acute illness, and cases in which the low vitamin B<sub>1</sub> excretion represents a real deficiency (Fig. 1). A vitamin B<sub>1</sub> tolerance test appears to be one way of solving this difficulty and work of this type is now in progress.

#### SUMMARY

A simple procedure for determining the urinary excretion of vitamin B<sub>1</sub> is described.

The accuracy of the method and its application to the study of vitamin B<sub>1</sub> metabolism in patients are discussed.

We wish to thank Dr. D. J. Hennessey, of the Department of Chemistry of Fordham University, for help in this work.

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## THE MEASUREMENT OF CELL VOLUME OF BLOOD BY THE CELL OPACITY METHOD\*

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THE problem of the volume of erythrocytes in blood is one of widespread interest. The variations under both physiologic and pathologic conditions have been widely studied for a long time. A great variety of methods have been devised for the estimation of cell volume. The data have been reviewed at length by Ponder<sup>1</sup> in his monograph, and by Guest<sup>2</sup> and Wintrobe.<sup>3</sup> Because of the difficulties involved in the various methods and the controversy over their accuracy, we have developed a new procedure which we have called the cell opacity method. This is based upon the principle that light is obstructed in proportion to the volume of cells present.

The transmission of light was applied to the measurement of cell volume following the suggestion of Dr. K. A. Evelyn, whose photoelectric colorimeter<sup>4</sup> offers a means of determining the opacity of blood cells with accuracy, ease of manipulation, speed and economy of material. Blood is collected in a micro-pipette and suspended in a citrate solution. It is then exposed to light passed through a filter which transmits light in the region of 660  $m\mu$ . The amount of light transmitted is measured by its effect on a photoelectric cell and recorded on a galvanometer. The obstruction of light by particles in suspension is a very complicated phenomenon.<sup>5</sup> Certainly some light must be reflected, refracted, and absorbed. The size and shape of the particles affect the transmission of light. It appears, however, that blood constitutes a system sufficiently constant that an empirical relationship may be established between cell volume and galvanometer readings. Although interference with the transmission of light is in itself a basic physical phenomenon of undoubted significance, this paper deals only with the correlation of the degree of opacity with cell volume.

Preliminary studies showed a direct relationship between cell opacity and the volume of blood used. In order to interpret the values obtained by the photocolormeter, it was necessary to make measurements of cell volume on the same blood by another method. Of all the methods described, the hematocrit method, in which the relative volume of blood cells and plasma is determined by centrifugalization, is in most general use. This method was accepted for this study as the standard of reference, and an extensive series of comparisons was made.

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## TECHNIQUE

The procedure adopted for the hematocrit method is as follows: Two to 3 c.c. of blood are obtained by venepuncture or heel stab and are either oxalated or heparinized to prevent coagulation. The oxalate solution contains 3 per cent ammonium oxalate and 2 per cent potassium oxalate; 0.04 c.c. per cubic centimeter of blood gives a final concentration of 0.2 per cent of oxalate.<sup>6</sup> The desired amount of solution is evaporated on the walls of a calibrated tube in which the blood is to be collected, so that the blood will not be diluted. If heparin is used, 1 mg. per cubic centimeter of blood is required. After mixing, the uncoagulated blood is placed in a Wintrobe tube<sup>7</sup> or a Rourke-Ernstene tube,<sup>8</sup> capped to prevent evaporation, and centrifuged at 3,000 r.p.m. for thirty minutes.

Centrifugalization causes a sharp separation of erythrocytes and leucocytes from the plasma. The volume of red blood cells alone or of the combined red and white blood cells is then read in per cent of the total volume. Inasmuch as the white cells ordinarily occupy a volume of about 0.5 per cent of the blood, for most purposes it is immaterial whether one reads the red cell volume or the total cell volume. In this study the volume of the erythrocytes alone was used.

The technique for the cell opacity method, adopted after considerable trial, is as follows: For this determination alone blood may be obtained without anticoagulants by skin puncture. If, however, a comparison is to be made with the hematocrit method, a portion of the blood containing anticoagulant, as described above, is used. Twenty cubic millimeters of blood are measured in a micro-pipette, calibrated "to contain." Ten cubic millimeters may be used, and the cell volume may be interpreted from the same chart if the result is multiplied by two. It is then delivered into a tube which contains 8 c.c. of citrate solution, and the pipette is rinsed with the solution.\* The tubes used must be of a size suitable for the Evelyn colorimeter, and tested for uniformity. The citrate solution contains 3.0 per cent of sodium citrate (41.5 Gm. per liter of sodium citrate  $.5\frac{1}{2}$  H<sub>2</sub>O). Before this solution is diluted to the final volume, 1 c.c. of formalin (36 to 38 per cent formaldehyde gas) is added for each liter of solution.

A tube containing 8 c.c. of the citrate solution is put into the photoelectric colorimeter with filter "660" of Evelyn. The setting is adjusted to give a galvanometer reading of 100. The tube containing the blood in citrate solution is then rotated to give an even suspension and is substituted for the "blank" tube. The galvanometer reading is not very steady at first, and deflects toward a lower number. After fifteen to thirty seconds it becomes quite constant and does not alter for a number of minutes. The value is then translated into terms of cell volume by reading from a plot on semilogarithmic paper, an alignment chart, or a table, according to convenience (see Fig. 1 and Table I). The establishment of these standards is described below.

## RESULTS

The method has a high degree of reproducibility. Because the procedure is so easy to carry out, 15 determinations were made on aliquots of the same

\*The colorimeter available at present is designed for either 6 or 10 c.c. of solution. To obtain the dilution given (1/400) it is necessary to use 25 c. mm. of blood. If 20 c. mm. are used in 10 c.c. of diluent, the values given for cell volume must be multiplied by  $\frac{5}{4}$ . If 10 c. mm. are used in 6 c.c. of diluent, the values given must be multiplied by  $\frac{5}{3}$ .



blood. The galvanometer readings did not vary more than half a point from the average in any sample.

We have made numerous comparisons of the values obtained by the hematocrit method and the cell opacity method with both normal and abnormal bloods. In this communication we report the results of only one series of determinations.

Through the kindness of Dr. John P. Hubbard and Miss Virginia Vogel, we were able to make observations on the blood of children attending the rheumatic heart clinic of the Children's Hospital. Many of the cases were convalescent or quiescent, and none were bed patients. Each child was subjected to venepuncture without undue stasis, and the blood was delivered from an all-glass syringe directly into a tube containing dry oxalate as described above.

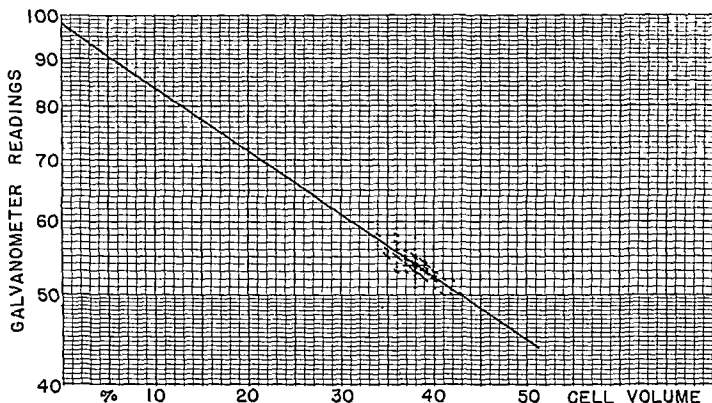


Fig. 1.—For description see text.

A series of 152 consecutive observations on blood samples examined for both cell volume and cell opacity is presented. No determinations were rejected. Refinements in technique for either method were not attempted; the hematocrit readings were recorded to the nearest half per cent of the volume of blood, and the cell opacity measurements to the nearest half mark on the galvanometer. These two values were plotted on semilogarithmic paper (see Fig. 1). The ordinates represent the logarithm of the galvanometer reading, and the abscissas represent the cell volume in per cent as determined by the hematocrit method. The point of intersection of the average cell volume and the average galvanometer reading was plotted and a line was drawn through this point to 98 on the ordinate. (With 100 per cent plasma and no cells the average colorimeter reading is 98.)

When the points showing the relationship of the cell opacity to the cell volume by the hematocrit method are plotted for each of the 152 cases, they are distributed very closely about the line in most cases, as shown in Fig. 1. Many of the points are superimposed and hence obscured, so the chart gives only an idea of the scatter.

A frequency histogram was then made to evaluate the divergence between the cell volume and cell opacity calculated as cell volume. In 51 cases the cell volumes derived from cell opacity measurement were in agreement with the hematocrit values within 0.5 volumes per 100 volumes of blood. In 32 and 34 cases, respectively, the cell opacity measurements showed a deviation of +1 and -1; in 10 and 13 cases, +2 and -2; in 4 and 3 cases, +3 and -3; in 2 and 3 cases, +4 and -4 volumes per 100 volumes of blood. No single determination gave a wider variation than this. These data when plotted (not given) show that the plus and minus errors form a close approximation to the probability curve of casual errors, and that over two-thirds of the cases are in agreement  $\pm 1$  volume per 100 volumes of blood, or within 2.5 per cent of the cell volume. According to Ponder, the hematocrit method is always correct  $\pm 5$  per cent, and most investigators claim a probable accuracy of 2 to 3 per cent. Inasmuch as the total variation found when the two methods are compared lies within the error of the hematocrit method alone, it is impossible to state the exact accuracy of the cell opacity method. It seems warranted to draw the conclusion that *in relatively normal cases* the accuracy of the cell opacity method approaches 1 per cent. Therefore, the values given in Fig. 1 and Table I may be considered valid as a standard for interpretation of cell opacity measurements in terms of cell volume.

TABLE I

CELL OPACITY GALVANOMETER READING	CELL VOLUME PER CENT	CELL OPACITY GALVANOMETER READING	CELL VOLUME PER CENT
85	9.5		
84	10.1	64	27.2
83	10.8	63	28.2
82	11.6	62	29.2
81	12.4	61	30.2
80	13.2	60	31.2
79	14.0	59	32.3
78	14.8	58	33.4
77	15.6	57	34.5
76	16.4	56	35.6
75	17.2	55	36.8
74	18.0	54	38.0
73	18.9	53	39.2
72	19.8	52	40.4
71	20.7	51	41.7
70	21.6	50	43.0
69	22.5	49	44.3
68	23.4	48	45.6
67	24.3	47	46.9
66	25.2	46	48.3
65	26.2	45	49.7

## DISCUSSION

*Hematocrit Method.*—The hematocrit method which has been used as a standard for comparison with the cell opacity method is itself open to criticism. The disadvantages of this method for clinical use are that, compared to the cell opacity method, it is time-consuming and laborious and requires a considerable amount of blood. It is often inconvenient and difficult to obtain so much blood from infants. It is highly desirable that cell volume be measured without resort

to venepuncture, and on small samples such as can be obtained from a finger prick or heel stab (or from small animals). Moreover, there is no general agreement as to the correct procedure even with this simple method. Different sizes and shapes of tubes have been employed. Various anticoagulants are said to cause swelling or shrinking of the cells. Different speeds and the duration of centrifuging also give variable results. Ponder states that various bloods act differently, so that the "correct" speed and time differ. In general, it has, however, come to be accepted that when blood is centrifuged at 3,000 r.p.m. for 30 minutes, the cells occupy a volume which can be called the cell volume. This value has been considered as accurate enough for clinical purposes, except in the study of special problems.

Under normal conditions, when the values obtained by the cell opacity and the hematocrit methods do not agree, the divergence is slight and the major error probably lies in the hematocrit determination. In any event the findings of the cell opacity method do not stand or fall by comparison to the hematocrit method, but may equally well be correlated with any other method for cell volume, and the values interpreted according to the standard adopted.

*Leucocytes.*—The cell opacity method registers the obstruction to the passage of light of all the components of blood. It has already been shown that the plasma alone exerts a small but definite effect on the transmission of light. The effect of hemoglobin is discussed below. In this paper we have correlated the opacity with the erythrocyte content of the blood only, but obviously the leucocyte content must also be taken into account. Ordinarily there are approximately 1,000 times as many red cells as white cells in the blood. Therefore, if the passage of light depended upon the number of cells alone, the error introduced by neglecting the white cells would be only 0.1 per cent. Owing to the larger size of the white cells, their volume is normally of the order of 1 per cent of that of the red cells. Preliminary observations have shown, however, that the opacity of a white cell emulsion corresponds more nearly to that of an equal number of red cells than to an equal volume of red cells. In any event the correction due to the white cells is so small as to be within the experimental error, and if our preliminary observations are correct, without any possible significance, except in extreme cases of leucocytosis. With refinements of technique or for special purposes, a standard could be constructed with a correction factor based upon the volume or number of the leucocytes.

In the unusual or rare case in which white cells are greatly increased, as in leucemia, caution should be used in application of the cell opacity method for measurement of cell volume. Too few cases are available at present for accurate analysis of this factor. When the leucocytes are increased to approximately half a million per cubic millimeter and the erythrocytes are diminished to two to three million, the volume of the white cells may be more than one quarter that of the red cells. Under such conditions the value for cell volume, as interpreted by the chart, will be greater than that for the red cells alone, but less than that for the total cell volume—red and white cells combined.

*Erythrocytes.*—Inasmuch as the opacity of blood is dependent mainly upon the erythrocytes, their variations must be taken into account. The two main variants of importance for discussion are number and size.

Numerous determinations of cell opacity were made, using different concentrations of blood in the final dilution. The results, interpreted in terms of volume, are directly proportional to the amount of blood used, when the concentration is not too great. The measurement of cell opacity is most accurate for cell volumes which lie in the normal or lower ranges. When the cell volume is above 45 per cent, as for example in the blood of patients with congenital heart disease and marked cyanosis, the values obtained by the measurement of cell opacity become slightly lower than those obtained by the hematocrit method—1 to 5 per cent. Apparently a greatly increased number of cells of normal size per unit volume of diluent introduces changes in the optical properties of the fluid. The practical solution is, however, simple. When the cell volume is greater than 45 per cent, one needs only to take a smaller sample of blood, e.g., 10 c. mm., or to add an additional 8 c.c. of diluent, and multiply by two the value obtained from the chart.

We have observed a few cases of hyperchromic anemia and erythroblastosis in which the red cells are of abnormally large size, or are both large and nucleated. The cell volume, as interpreted from the cell opacity method, is lower than that obtained by the hematocrit method, 3 to 15 per cent. Whether this divergence is due primarily to the increase in cell size, the variability in cell size, or to some particular element in the blood has not yet been determined. We have not studied a sufficient number of cases to warrant discussion of the effect of variation of the shape and size of erythrocytes within a single blood (anisocytosis and poikilocytosis).

Microcytosis, conversely to macrocytosis, produces cell opacity values which are interpreted as 1 to 3 per cent too high, according to the hematocrit standard. This finding is in accordance with the studies of Drabkin and Singer<sup>5</sup> which show that obstruction of light is partly dependent upon particle size. We have encountered no case in which the divergence is sufficient to be clinically significant. In case of doubt the determination should be checked by the hematocrit method.

In addition, the factors of cell size and cell number are interrelated. Rat blood contains about twice as many erythrocytes as human blood, but because the cells are only half as large, the cell volume is nearly the same, and the cell opacity shows only slight divergence from that of human cells.

*Hemoglobin.*—Drabkin and Singer<sup>5</sup> showed that the transmission of light through erythrocytes is affected by the hemoglobin content. The factor for hemoglobin varies with the wave length used, as can be postulated from the absorption curve. They did not report observations beyond 630  $m\mu$ . At this point the effect of hemoglobin was of the order of 1 per cent. We selected a filter which transmits light at or near 660  $m\mu$  for our study primarily because it is not sensitive to differences in hemoglobin.\* This may be proved by adding saponin and ammonia to the sample and the blank. The hemolyzed blood thus obtained gives a galvanometer reading for opacity hardly greater than for plasma alone. The hemoglobin concentration, therefore, has no effect on the determination.

\*In a personal communication Dr. Drabkin states that the measurements he has made at 660  $m\mu$  show that the effect of hemoglobin is negligible at this wave length.

*Diluent.*—Formalin was added to the citrate solution at the suggestion of Dr. Evelyn, who recommended it as giving greater uniformity and reliability to the measurement. This has been our experience also. Apparently the formalin affects the size or shape of the cells, for when it is omitted the values for cell volume calculated from the chart are diminished about one-fourth. Presumably it makes the cells more plump, but it does not alter their ability to swell or shrink.

When hematocrit determinations are made, the anticoagulant used is of importance in either increasing or decreasing the measured cell volume. In the cell opacity method, however, the effects of the anticoagulant are so minimized by the four hundredfold dilution which the blood undergoes that they need not be considered.

The blood cells vary in volume primarily with the osmotic pressure, and secondarily, with the acid-base equilibrium of the fluid in which they are suspended. Hypertonic and alkaline solutions dehydrate the cells and cause shrinkage. Hypotonic and acid solutions cause an imbibition of water and swelling of the cells. It might, therefore, be questioned whether the solution used as a medium for suspension of the cells rigidly meets the requirements of isotonicity and correct pH. This need not be discussed, however, because the answer is not relevant to the problem. The actual osmotic pressure and acidity of the diluent used are not of prime importance because all bloods are treated alike. Whether or not the diluent is isotonic, the cells are still capable of swelling and shrinking. If a diluent of different osmotic pressure and pH were used, the results should still be proportional to the volume of red cells in the blood sample. It would be necessary only to determine the relationship under the altered conditions, between the degree of opacity and the results of an accepted method for determining cell volume.

Whatever the absolute size of the cell may be under the given conditions is unimportant. It is important, however, that it should be affected in a constant and reproducible manner. The relationship of the transmission of light and the observed hematocrit value is an empirical one; the correlation of the values, as determined by the two methods, is all that is required. This agreement has been demonstrated, as presented above.

*Significance of the Method.*—Other methods which deal with the blood under more or less physiologic conditions of carbon dioxide and oxygen tension attempt to measure the actual percentage volume of the cells. The cell opacity method, because of the large dilution of the blood, may be said to eliminate differences in volume due to the original condition of the cells *in vivo* with regard to oxygenation or acid-base equilibrium of the plasma. This method may be regarded as measuring the cell volume under *standardized conditions*.

In certain pathologic conditions the results of the cell opacity method may not be in agreement with those of the hematocrit method. Any difference may at first appear to be due to a defect inherent in the cell opacity method. Such is not the case. Whereas ordinarily one would be wrong and the other right, these two methods measure different physical properties of the blood, and, therefore, both may be valid. Furthermore, the difference in the results obtained

by the two methods on pathologic bloods is in itself a significant datum, and may come eventually to be correlated with a difference in the state of the cells which cannot be described in terms of either method alone.

#### SUMMARY AND CONCLUSIONS

A single drop of blood is sufficient to determine the transmission of light (660  $m\mu$ ) through a blood suspension in citrate solution.

This measurement is directly correlated with the value for cell volume determined by the hematocrit method.

Alteration in size, shape, and structure of erythrocytes causes a demonstrable deviation from the values obtained by the hematocrit method, but for nearly normal cells the accuracy of the cell volume by the cell opacity method is greater than that of the hematocrit method.

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#### A SIMPLE PERFUSION PUMP\*

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SINCE the advent of Lindbergh's "Mechanical Heart" many physiologists have had the desire to work with this apparatus. However, the expense connected with its construction made it, in many instances, an impossible acquirement. Therefore, a plan is presented for a perfusion pump which is inexpensive and has been found satisfactory for perfusing the organs of rats, guinea pigs, and rabbits.

The apparatus, shown on Fig. 1, is constructed entirely of pyrex glass, and all connections are ground glass. The artificial media for perfusion have been well put forth by Parker<sup>2</sup> and have been found very satisfactory with this perfusion pump. To sterilize this apparatus it is best to separate the individual parts and wrap them in separate cloths. After sterilization is completed, the main chamber C is clamped to a stand and covered over with a sterile cloth

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until ready for the insertion of the organ to be perfused. The organ is dissected out of the animal under sterile conditions and washed with Tyrode's solution. The narrow end of the perfusion tube *B* is inserted into the main blood vessel of the organ and tied there with silk thread. There is a slight enlargement about 2 mm. from the delivery end of the perfusion tube to prevent the blood vessel from slipping once it is tied. The diameter of the delivery end of the perfusion tube ranges from 0.5 mm. to 3 mm., according to the size of the blood vessel.

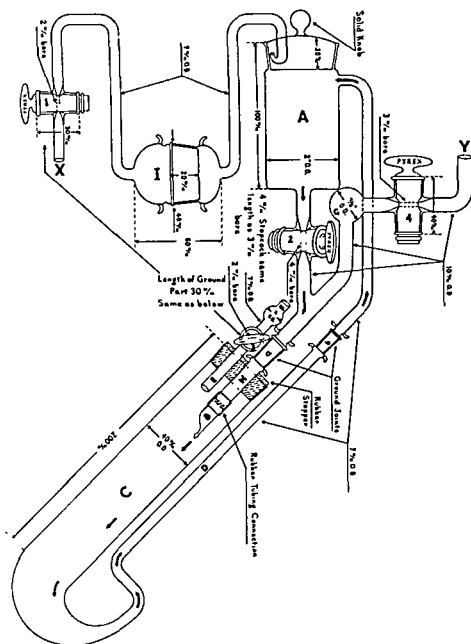


Fig. 1.—Sketch of perfusion pump showing dimensions and structural details.

A rubber bulb is filled with Tyrode's solution, attached to the wider end of the perfusion tube, and the solution is then forced through the blood vessels to clear them of blood. The rubber bulb is detached and the wider portion of the perfusion tube is inserted into the rubber tubing connection. The rubber connection is connected to the delivery tube *H* which runs through a rubber stopper. This rubber stopper is inserted into the main chamber *C*, and the reservoir *A* with its attached parts is connected at the ground glass connections *a* and *b*.

One hundred cubic centimeters of the perfusing medium is put into the reservoir *A* and then its ground glass cover is inserted in place. All stopcocks

are kept closed until operation is ready to begin. Drying chamber *I* is opened and filled with anhydrous calcium chloride and sterile cotton. The purpose of this chamber and its contents is to prevent any water vapor from coming in contact with the medium after the water suction, which is connected at *X*, has been on for a period of time. The drying chamber is closed; bulb *F* is packed loosely with sterile cotton, and the pump is ready for operation.

The entire apparatus is put into an incubator at about 37° to 39° C. and the suction at *X* is turned on. Stopcock 1 is opened halfway, allowing a slight suction to exert a pull in the reservoir *A*. Stopcock 3 is opened about halfway, so that air will enter the main chamber *C* and thus prevent the accumulation of too high a vacuum in the main chamber which would cause a back-pull on the perfusion tube. Stopcocks 2 and 4 are opened, and the air is forced out through opening *Y* which has a plug of cotton in it. The medium now flows down by gravity to the perfusing tube. After the air has been replaced by the medium, stopcock 4 is closed. Another purpose of this opening *Y* is to make possible the addition of substances without the necessity of opening the reservoir cover.

The force of gravity is sufficient to pull the fluids through the blood vessels. After the medium has passed through the organ, it flows down along the lower surface of the main chamber and as it reaches the lower portion of the bend, the suction at *X* is sufficient to draw the medium through the tube *D* and up into the reservoir chamber *A*. This circulation goes on continuously as long as there is suction at *X*.

#### SUMMARY

A perfusion pump is herein described that is simple in construction and has been found satisfactory for perfusing small organs.

NOTE.—Since this paper was written, a modification was made in the apparatus described here. In order to remove fluid from the pump for analysis a stopcock opening was made in the lower portion of reservoir *A*. It was also found that a small centrifugal pump inserted between ground glass joint *a* increased the perfusing efficiency of the pump.

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# DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

**PATCH TEST, A New Modification of (the Chamber Method),** Rokstad, I. Arch. Dermat. & Syph. 41: 649, 1940.

For examination of the nonspecific toxic (i.e., caustic) effect on the skin exerted by certain volatile substances (e.g., terpenes) the patch test method commonly employed in eczema is unreliable.

A new method has been worked out, therefore, in which the cellophane plate (or impregnated cambric) is replaced by a celluloid chamber which is fixed to the skin with adhesive tape (or, in cases of hypersensitiveness to the tape, with a paste made with 15 Gm. of zinc oxide, 15 Gm. of gelatin, 25 Gm. of glycerin, and 45 c.c. of distilled water).

When applied correctly, the chamber exerts a suction effect on the skin, not merely providing an airtight space that prevents escape of the volatile test substances, but inducing the formation of a papule within a certain length of time.

This papule, then indicates that the suction effect has been present; in other words, it constitutes the criterion of a successful test technique. Besides, the edematous condition of the papule may be assumed to facilitate the absorption of the test substances through the horny and sebaceous barrier of the skin.

In studies on the toxicity (i.e., the caustic effect) of the terpenes, which will be published later, this method has given constant results, revealing lower threshold values than had been previously established for erythematous or for bullous (purulent) reactions.

**LUNG, Clinical Studies of Primary Carcinoma of the,** Overholt, R. H., and Rumel, W. R. J. A. M. A. 114: 735, 1940.

Primary carcinoma of the lung is a relatively common disease which is responsible for approximately 10 per cent of all cancer deaths.

In this clinical study 75 patients had histologically proved primary carcinoma of the lung.

Partial or complete bronchial occlusion, with or without secondary infection, produces the most important pathologic lesions, giving rise to early symptoms, physical manifestations, and roentgenologic changes.

Bronchoscopic examination is by far the most important diagnostic procedure available and should be used without delay in the study of any case in which a dry or productive cough develops alone or in association with hemoptysis, wheezing, dyspnea, chest discomfort, or other symptoms suggestive of early primary carcinoma of the lung that cannot be definitely explained on some other basis.

The diagnosis was established with histologic verification at an operable stage in 24 per cent of the 75 cases.

Lobectomy and pneumonectomy are practical therapeutic procedures, without an excessive operative mortality rate, which at the present time appear to offer a good chance of survival to the patient suffering from primary carcinoma of the lung.

**TISSUE, Comparison of Starch Paste and Albumin Mixture as Agents for the Routine Mounting of Paraffin Sections,** McDowell, A. M., and Vassos, G. A., Jr. Arch. Path. 29: 432, 1940.

The albumin-glycerin mixture is made up by taking equal parts of egg white and glycerin, filtering, and adding a small crystal of thymol as a preservative. The starch paste tried is essentially the same as the one described by Spoerri. One gram of powdered starch is

added to 10 c.c. of cold water and thoroughly mixed; the solution is then poured into 20 c.c. of boiling water, 2 drops of dilute hydrochloric acid are added, and the suspension is boiled for five minutes while being constantly stirred to free the opalescent suspension from lumps of starch. A small crystal of thymol should be added after the paste has cooled.

The clean glass slides are prepared by coating one surface with a thin film of the mixture to be used. Drying of the affixative on the slides does not seem to be a disadvantage as long as no dust is allowed to collect on them.

Paraffin sections are then flattened out on the surface of warm water and floated onto the slides already smeared with the adhesive to be used. Sections thus mounted on slides are ready for drying prior to their deparaffinization and staining.

The table shows the method of drying adapted to varied staining techniques.

DRYING METHOD	AFFIXATIVE	MODIFIED MASSON STAIN	HEMA- TOXYLIN- EOSIN	MODIFIED GIEMSA	FOOT SILVER FOR BRAIN	ZIEHL- NEELSEN	FOOT SILVER FOR RE- TICULUM
On hot plate for 3 minutes and in drying oven at 56° to 58° C. for 1 hour	Albumin Starch	A A	A A	A A	A A	A I	I I
In drying oven at 38° to 40° C. for 24 hours	Albumin Starch	A A	A A	A A	A A	A A	I I
In drying oven at 56° to 58° C. for 48 hours or at 38° to 50° C. for 5 days	Albumin Starch	U U	U U	U U	U U	U U	A A

A = adequate; I = inadequate; U = unnecessary.

#### PROTHROMBIN, in the Blood of Newborn Mature and Immature Infants, Kato, K., and Poncher, H. G. J. A. M. A. 114: 749, 1940.

The prothrombin clotting time of 173 newborn infants, both mature and immature, has been determined by the microprothrombin method; an aggregate of 1,595 tests were made during the neonatal period, extending over four weeks of postnatal life.

The average prothrombin time of 100 mature infants on the first day of life was found to be 43.2 seconds; gradually shortening as the infants grew older, by the tenth day of life the average time was twenty-five seconds.

The average prothrombin time of 73 premature infants was 46.5 seconds on the day of birth and showed a much greater degree of fluctuation on subsequent days than that of mature babies. Also in this group were some infants whose prothrombin time actually increased after birth; in one instance the prolonged prothrombin time of the blood was associated with strangulated hernia.

No correlation was found to exist between the degree of the infant's maturity and the prothrombin clotting time of the blood, at least on the day of birth.

The low prothrombin level of the blood in newborn infants satisfactorily explains the pathogenesis of hemorrhagic disease of the newborn. This may be expressed clinically as hemorrhage into the gastrointestinal tract (melena, hematemesis) or bleeding from the cord (omphalorrhagia) or genitourinary tract (hematuria). While in some cases of intracranial hemorrhage birth trauma was the precipitating factor, the severity of the bleeding was apparently induced by a lowering of the prothrombin level of the infant's blood.

#### GLUCOSE TOLERANCE Test, Interpretation of, Conn, J. W. Am. J. M. Sc. 199: 555, 1940.

A short period of carbohydrate restriction in the normal person causes a marked delay in the rate of utilization of carbohydrate, as indicated by the glucose tolerance test.

Undernourished individuals respond to the same procedure with a greater loss of carbohydrate tolerance.

Erroneous diagnoses of diabetes mellitus are made when the factor of previous carbohydrate restriction is neglected.

Unless the influence of previous carbohydrate restriction is removed by means of an adequate preparatory diet, the glucose tolerance test is not reliable as an indicator of the individual's ability to utilize carbohydrate.

**SULPHANILAMIDE, Significance of the Oxidation of, During Therapy, Fox, C. L., Jr.**  
Am. J. M. Sc. 199: 487, 1940.

The conclusion that sulfanilamide is oxidized during therapy is supported by these observations:

1. Complete, initial deprivation of oxygen interferes with bacteriostasis.
2. Increased oxygen availability magnifies bacteriostasis.
3. Reducing agents and large inocula, which rapidly reduce the potential, obliterate bacteriostasis despite excess of sulfanilamide.
4. The use of a preformed oxidant derived from sulfanilamide, 4-hydroxylamino sulfonamide, eliminates the usual initial growth period before bacteriostasis appears and gives a more prolonged effect than sulfanilamide.
5. The occurrence of methemoglobinemia during therapy and the occurrence of an oxidation product of sulfanilamide in the urine of treated patients confirm the view that an oxidant is produced in the blood or tissues of the patient.

**SYPHILIS: Evaluation of the Spirochete Complement Fixation Reaction in Comparison With the Eagle Flocculation and Wassermann Procedures, Erickson, P. T., and Eagle, H.** Ven. Dis. Inform. 21: 39, 1940.

In confirmation of Gaetgens' findings, phenolized suspensions of the Reiter strain of cultured spirochetes (palligen) react with syphilitic serum to give positive complement fixation, and the results compare favorably in both sensitivity and specificity with standard Wassermann and flocculation procedures.

In 490 sera from a group of syphilitic patients under treatment or observation, a spirochetal complement fixation technique detected 85 per cent, as compared with 68.5 per cent for an icebox Wassermann test, and 75.5 per cent for the Eagle microflocculation procedure.

Of 542 patients from a general dispensary and hospital population, with initially negative Wassermann and flocculation tests, there were 18 in whom the spirochetal complement fixation test was positive or doubtful. In no less than 14 of these could a definite diagnosis of syphilis be made on the basis of history, clinical evidence, or subsequent serologic findings.

In 88 spinal fluids the Wassermann and spirochetal complement fixation tests were both negative in 60 and both positive in 26. In 2 cases the spirochetal test only was weakly positive, and in one of these the history and clinical findings furnished strong presumptive evidence of central nervous system syphilis.

The fact that the spirochetal concentration which must be used in the test is only one-third or one-fourth the anticomplementary concentration is an obvious drawback to the routine use of the test.

**BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D.** J. A. M. A. 114: 850, 1940.

Progressive hemolysis occurs in human blood stored in any of the preservatives studied.

The rate of hemolysis is much greater when blood is stored at 20° C. than at 5° C.

The addition of large quantities of isotonic dextrose solution to blood slows considerably the rate of hemolysis as compared with that in blood stored with little or no added dextrose.

The Rous-Turner blood-dextrose-citrate mixture has been so modified that it can safely be given intravenously without discarding the plasma. This modification consists of 10 volumes of blood, 13 volumes of 5.4 per cent aqueous solution of anhydrous dextrose, and 2 volumes of 3.2 per cent dihydric sodium citrate in water.

Blood stored in the modified blood-dextrose-citrate mixture will hemolyze only one-twenty-fifth to one-fiftieth as much in thirty days at 5° C. as will blood in sodium citrate alone or in sodium citrate plus sodium chloride.

The blood-dextrose-citrate mixture has proved safe and practical for human transfusions and is of distinct advantage in the operation of blood banks with a small volume of transfusions.

Hemolysis is less in blood stored in sealed flasks from which the air is completely displaced by the blood mixture than it is in blood exposed to the air.

Blood stored in flasks containing air trapped by rubber stoppers hemolyzes no faster than blood exposed to the air in cotton-plugged flasks.

Erythrocytes stored in the dextrose-citrate mixture resist destruction by shaking better than do those stored in sodium citrate alone or in citrate-saline mixtures.

The initial hemolysis encountered when blood is drawn into large volumes of preservatives can be prevented if the blood is cooled rapidly and uniformly by mixing it with preservatives which have been previously cooled to about 5° C.

**BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. 114: 855, 1940.**

There is progressive diffusion of potassium from human erythrocytes into the plasma during storage.

This diffusion is rapid during the first five days but becomes gradually less. A maximum concentration of potassium in the plasma is approached in from fifteen to twenty days.

The high values of plasma potassium attained in from fifteen to thirty days of storage cannot be accounted for by the release of that ion from completely hemolyzed cells.

Variations in the content of sodium, chloride, citrate, and dextrose in the preservative mixtures or storage at different temperatures and in various atmospheres did not affect the diffusion rate.

**BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. 114: 858, 1940.**

The high plasma potassium content in blood preserved for thirty days is not toxic when the blood mixture is transfused into human beings at velocities of less than 43.3 c.c. per minute. The concentrations of plasma potassium encountered in blood stored for one month are not high enough to cause significant increases in the serum potassium of the recipient.

**BLOOD, Studies on Preserved, DeGowin, E. L., Harris, J. E., and Plass, E. D. J. A. M. A. 114: 859, 1940.**

A detailed study of ten patients who were given transfusions of blood mixtures varying from 25° to 15° C. and at velocities from 6.0 to 42.8 c.c. per minute revealed no significant lowering in the body temperature, no consistent changes in blood pressure, and no untoward clinical symptoms.

Preserved blood has been administered without preheating in 568 cases, with no reactions which could be attributed to the procedure.

The practice of transfusing preserved blood cold is a great convenience in conserving time and prevents untoward reactions involved in the process of heating it.

Parenteral fluids other than blood can be administered at room temperature with safety.

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